

POWDER FORMULATIONS OF rSEB FOR IMPROVED VACCINATION

This application claims priority to U.S. Provisional Patent Application number 60/448,109 filed February 20, 2003, which is hereby incorporated by reference in its entirety.

BACKGROUND

Field of the Invention

[0001] The present invention relates, *e.g.*, to compositions of dried pharmaceuticals, in particulate (*e.g.*, in powder) form. Such compositions are suitable for reconstitution and parenteral administration (*e.g.*, subcutaneous, intravenous, intramuscular and intradermal delivery) or direct administration of powder to mucosal tissues (*e.g.* intranasal administration). Compositions prepared by the methods of the invention, and methods of administering the compositions to a patient, are also described. Exemplary inventive compositions include recombinant Staphylococcal Enterotoxin B Vaccine (rSEB).

Background Information

[0002] Methods have been reported for formulating dried pharmaceutical compositions. These methods include, *e.g.*, steps of precipitation, spray-drying, and/or mechanical milling of dried substances. Some of the reported methods utilize non-aqueous solvents to provide rapid moisture evaporation and to reduce processing time. Such solvents can damage the pharmaceutical agents (*e.g.*, proteins) being dried. Particles produced by the reported methods often exhibit a tendency to agglomerate, and/or lack a suitable size, density (*e.g.*, tap density), morphology and/or stability for optimal pharmaceutical use.

[0003] There is a need for methods to produce dried pharmaceutical compositions that lack one or more, or other, of the above-mentioned drawbacks.

SUMMARY OF THE INVENTION

[0004] The present application relates, *e.g.*, to pharmaceutical compositions in the form of powders, produced by drying a liquid formulation containing an active pharmaceutical. In

another embodiment, the invention relates, *e.g.*, to a method of preparing a pharmaceutical composition in particulate form, (*e.g.*, in the form of a powder), reconstituting to form a solution, and delivery of the solution parenterally to achieve a protective immune response. Such pharmaceutical compositions, methods of making the pharmaceutical compositions and methods of using the compositions are described.

BRIEF DESCRIPTION OF THE DRAWINGS

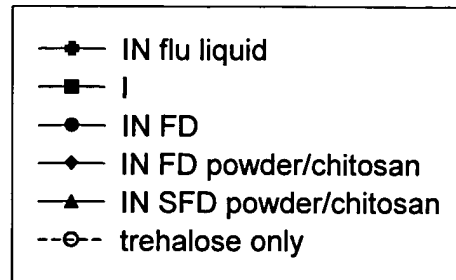
[0005] Various features and attendant advantages of the present invention will be more fully appreciated as the same becomes better understood when considered in conjunction with the accompanying drawings.

[0006] Fig. 1A shows a schematic view of a spray-freeze atmosphere dry apparatus of the invention.

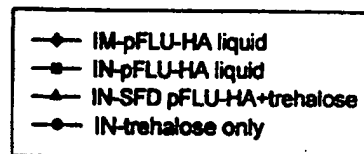
[0007] Fig. 1B shows a schematic view of a spray-freeze-drying set-up with Vibration and Internals.

16.	SFD chamber	36.	Pump
2.	Spray nozzle	20.	Cooling system
48.	Heating tape	44.	By-pass valve
12.	Solution (liq.)	46.	By-pass line
28.	Filter	18.	Nebulizing air
38.	Valve	49.	Vibration source
32.	Air Filter	50.	Internals

[0008] Fig. 2 shows the serum antibody (Ab) response following IN delivery of various flu vaccine formulations.



[0009] Fig. 3 shows the serum Ab response rats following immunization with pFLU-HA.

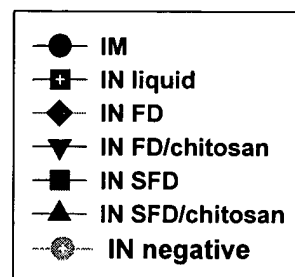


[00010] Fig. 4 shows a particle size distribution liquid virus particles produced by an Accuspray™ nozzle, as measured by laser diffraction.

[00011] Fig. 5 shows luciferase gene expression after IN liquid pCMV-LUC delivery.

[00012] Fig. 6 shows luciferase gene expression in rats after IN pCMV-LUC delivery.

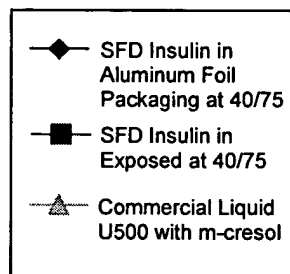
[00013] Fig. 7 shows serum Ab titers following pFLU-HA immunization.



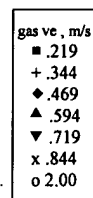
[00014] Fig. 8 shows a scanning electron microscope (SEM) image of SFD insulin sprayed through an Accuspray™ nozzle and dried by lyophilization.

[00015] Fig. 9 shows a scanning electron microscope (SEM) image of the SFD insulin shown in Figure 9, but at a higher magnification.

[00016] Fig. 10 shows desamido (chemical degradation) detected for SFD and liquid insulin samples, as a measurement of stability.



[00017] Fig. 11 shows the moisture and drying time of a composition produced by a freeze dry atmosphere method.



[00018] Fig. 12 shows an SEM image of mannitol powders produced by the spray-freeze-atmospheric drying process.

[00019] Fig. 13A shows a comparison of serum immune responses following IN delivery of SFD flu vaccine with and without chitosan.

[00020] Fig. 13B shows a comparison of nasal mucosal immune responses following IN delivery of SFD flu vaccine with and without chitosan, on day 56.

[00021] Fig. 14 shows the moisture levels of samples collected from the top and bottom of a fluidized bed, from an integrated SFD process without vibration and internals (low drying gas velocity (.39 m/s).

[00022] Fig. 15 shows an SEM of particles sampled at 30 minutes from the top of a fluidized bed.

[00023] Fig. 16 shows an SEM of particles sampled at 60 minutes from the top of a fluidized bed.

[00024] Fig. 17 shows the porosity of powders dried at two different gas velocities. Shown is the effect of flow-rate on sublimation time (V-FB-SFD).

[00025] Fig. 18 shows particle size distribution for mannitol particles obtained by spray-freezing through an Accuspray™ nozzle and drying by lyophilization. The particle size distribution was measured by laser diffraction.

[00026] Fig. 19 shows a scanning electron microscope (SEM) image of SFD neat insulin powders (which are more resistant to moisture than insulin/lactose).

[00027] Fig. 20 shows an SEM of SFD insulin/lactose composite powder after exposure to ambient moisture.

[00028] Fig. 21 shows capsules with SD (spray-dry) and SFD (spray-freeze-dry) powders after rupturing the capsule membranes. There is no visible lactose remaining in the capsule with the SFD powder.

[00029] Fig. 22 shows a Fourier transform infrared (FTIR) spectrum for liquid rSEB vaccine 10 mg/ml in PBS.

[00030] Fig. 23 shows an FTIR spectrum for Lyophilized rSEB Vaccine in Sucrose.

[00031] Fig. 24 shows an FTIR spectrum of the ALP rSEB Vaccine in Sucrose.

[00032] Figure 25 shows the results of immunoassay testing, represented as a plot of the estimated normalized treatment differences between SFD rSEB, lyophilized rSEB, and unprocessed rSEB.

DESCRIPTION OF THE INVENTION

[00033] The present invention relates, *e.g.*, to methods of preparing dried pharmaceutical compositions, in particulate form (*e.g.*, in a powder); to compositions made by these methods; and to methods of using the compositions to treat patients.

[00034] One aspect of the invention is a method of preparing a pharmaceutical composition, comprising one or more of the following steps: atomizing a liquid formulation of a therapeutic or prophylactic agent to produce an atomized formulation; freezing said atomized formulation to form solid particles; and drying said solid particles to produce dried particles (*e.g.*, a powder). Preferably, said atomized formulation comprises droplets having a volume mean diameter (as defined by W.H.Finley, "The mechanics of inhaled pharmaceutical aerosols, an introduction", Academic Press, London, UK (2001)) of between about 35 μ m and about 300 μ m, more preferably between about 50 μ m and about 300 μ m, and/or said powder comprises dried particles having a volume mean diameter of between about 35 μ m and about 300 μ m, more preferably between about 50 μ m and about 300 μ m. Most preferably, these droplets or particles have a volume mean diameter of between about 50 μ m and about 100 μ m. In a preferred embodiment, at least about 50% of the dried particles have a volume diameter within about 80% of the mean; more preferably, at least about 50% of the dried particles have a volume diameter within about 60% of the mean. In a preferred embodiment, the powder comprises dried particles that have a mean aerodynamic diameter (as defined in W.H. Finley, *supra*) of between about 8 μ m and about 140 μ m, more preferably between about 8 μ m and about 80 μ m, still more preferably between

about 20 μ m and about 70 μ m. This method, and compositions made by the method, are sometimes generally referred to herein as a “spray-freeze-dry” method or compositions.

[00035] Particles of the above pharmaceutical compositions are of an appropriate size, density and/or morphology to facilitate reconstitution and parenteral administration or intranasal administration in dry form. Without wishing to be bound by any particular theory, it is proposed that, following intranasal administration, the compositions described above are delivered to mucous membranes, *e.g.*, of the nasal lining or the sinuses, where they adhere, rather than being propelled through the sinus cavities into the pulmonary system, and that adherence to such mucous membranes allows a faster rate of absorption than with other formulations of therapeutic and prophylactic compositions. When the inventive composition is a vaccine, an enhanced antibody response is produced, thus providing improved protection.

[00036] Another aspect of the invention is a method prepare a pharmaceutical composition, comprising one or more of the following steps: atomizing a liquid formulation of a therapeutic or prophylactic agent to produce an atomized formulation; freezing said atomized formulation to form solid particles; and drying said solid particles at about atmospheric pressure, in the presence of vibration, internals, mechanical stirring, or a combination thereof, to produce dried particles (*e.g.* to produce a powder). By “about atmospheric pressure” is meant herein a pressure ranging from about one half atmosphere to about five atmospheres. By “drying” is meant herein removal of the volatile components of the formulation from the solid frozen particles. Preferably, the powder comprises dried particles having a volume mean diameter of between about 35 μ m and about 300 μ m, more preferably between about 50 μ m and about 300 μ m, or most preferably between about 50 μ m and about 100 μ m; and/or the dried particles have a volume mean aerodynamic diameter of between about 8 μ m and about 140 μ m, preferably between about 8 μ m and about 80 μ m, more preferably between about 20 μ m and about 70 μ m. In a preferred embodiment, at least about 50% of the dried particles have a volume diameter within about 80% of the mean; more preferably, at least about 50% of the dried particles have a volume diameter within about 60% of the mean. Preferably, the frozen, solid particles are in a fluidized state as they are being dried. This method, and compositions made by the method, are

sometimes referred to herein as “spray-freeze-atmospheric-dry” method or compositions. An advantage of compositions produced by this method is that they do not agglomerate.

[00037] Other advantages of the compositions of the invention are that the compositions offer good stability and sterility, and are readily reconstituted in liquid. The particles may exhibit a low tap density, and high surface area, which facilitates the reconstitution of the particles. The particles exhibit low levels of fines, which renders them easy to handle. The methods of the invention allow for a well-controlled distribution of particle sizes. Therefore, the inventive compositions comprise a well-controlled distribution of particle sizes. The compositions are stable in the absence of refrigeration, allowing for more convenient and less expensive storage and transportation than, *e.g.*, liquid formulations. Compositions of the invention are particularly well suited for mass vaccinations. In one embodiment of the invention, inventive compositions are reconstituted in liquid and administered by intramuscular injection. In an alternative method, compositions of the invention may be administered intranasally, since the well controlled particle size distribution allows accurate targeting of the nasal mucosa.

[00038] The property of tap density is well known to those of skill in the art. Each particle of a solid material has the same true density after grinding, milling or processing, but the material occupies more geometric space. In other words, the geometric density is less, approaching 50% less, than the true density if the particles are spherical.

[00039] Handling or vibration of powdered material causes the smaller particles to work their way into the spaces between the larger particles. The geometric space occupied by the powder decreases and its density increases. Ultimately no further natural particle packing can be measured without the addition of pressure. Maximum particle packing is achieved.

[00040] Under controlled conditions of tap rate, tap force (fall) and cylinder diameter, the condition of maximum packing efficiency is highly reproducible. This tap density measurement is formalized in the British Pharmacopoeia method for Apparent Volume, ISO 787/11 and ASTM standard test methods B527, D1464 and D4781 for tap density.

[00041] Another aspect of the invention is method of making a pharmaceutical composition as above, wherein the freezing is performed by introducing the atomized formulation into a cold fluid or medium having a temperature below the freezing point of the liquid formulation (the term "a fluid" as used herein encompasses both a gas, such as a compressed gas, and a liquid); wherein said fluid or medium has a boiling point or sublimation point lower than that of the atomized formulation; wherein the drying is performed at about atmospheric pressure (preferably in the presence of vibration, internals, mechanical stirring, or a combination thereof), by lyophilization, or by a combination thereof, preferably wherein the freezing and drying are both performed in a cold gas at about atmospheric pressure (preferably in the presence of vibration, internals, mechanical stirring, or a combination thereof); wherein the therapeutic or prophylactic agent is a protein (*e.g.*, insulin), a nucleic acid or a virus particle, or wherein the therapeutic agent is an immunogenic agent, such as an influenza vaccine, *e.g.*, a vaccine that comprises inactivated influenza particles, a subunit influenza vaccine, or a nucleic acid encoding an influenza haemagglutinin protein, particularly wherein the haemagglutinin protein is under the control of a constitutive promoter, particularly a strong constitutive promoter such as a CMV promoter; wherein the liquid formulation further comprises a pharmaceutically acceptable excipient, such as a mucoadhesive, *e.g.*, chitosan, dermatan sulfate, chondroitin or pectin, or wherein the liquid formulation consists essentially of the therapeutic agent and water.

[00042] Another aspect of the invention is a pharmaceutical composition prepared by a method as above; or a pharmaceutical composition that comprises dried particles having a volume mean diameter of between about 35 μ m and about 300 μ m, preferably between about 50 μ m and about 300 μ m, more preferably between about 50 μ m and about 100 μ m and/or wherein the dried particles have mean aerodynamic diameter of between about 8 μ m and about 140 μ m, more preferably between about 8 μ m and about 80 μ m, still more preferably between about 20 μ m and about 70 μ m. Preferably, at least about 50% of the dried particles in the composition have a volume diameter within about 80% of the mean; more preferably, at least about 50% of the dried particles have a volume diameter within about 60% of the mean.

[00043] Another aspect of the invention is a method of treating a patient in need thereof, comprising administering to the patient an effective amount of a pharmaceutical composition produced by a method of the invention and/or a pharmaceutical composition having the properties noted in the preceding paragraph; wherein the composition is administered by a parenteral, respiratory, intranasal, intrarectal, intravaginal, or sublingual route. Another aspect is a method of reducing the amount of a therapeutic or prophylactic agent that is required to produce an efficacious result following intranasal administration to a patient in need thereof, comprising administering to the patient, intranasally, an effective amount of a pharmaceutical composition of the invention. Another aspect is a method of eliciting an immune response in a patient, comprising administering to the patient an effective amount of an immunogenic composition of the invention, *e.g.*, wherein the composition is administered directly as a powder to a mucosal surface or parenterally after reconstitution.

[00044] Another aspect is a method of preparing a pharmaceutical composition, comprising drying at about atmospheric pressure, in the presence of vibration, internals, mechanical stirring or a combination thereof, solid particles which have been formed by freezing an atomized formulation of a liquid formulation of a therapeutic or prophylactic agent.

[00045] Another aspect is a method of preparing a pharmaceutical composition, comprising atomizing a liquid formulation of said therapeutic or prophylactic agent to produce an atomized formulation, such that, following freezing of said atomized formulation to form solid particles, and drying of said solid particles to produce a powder, the dried powder comprises dried particles having an average mean size diameter of between about 35 μ m and about 300 μ m, preferably between about 50 μ m and about 300 μ m, and more preferably between about 50 μ m and about 100 μ m, wherein at least about 50% of said dried particles have a volume diameter within about 80% of the mean, and said dried particles having a mean aerodynamic diameter of between about 8 μ m and about 140 μ m.

[00046] Any of a variety of therapeutic or prophylactic agents can be used in the methods and compositions of the invention. A “therapeutic agent” (sometimes referred to herein as an “active

pharmaceutical agent” or API), as used herein, means an agent that can elicit a therapeutic effect in a cell, tissue, organ or patient to which it is administered. Compositions that comprise one or more therapeutic agents can produce a “clinically efficacious result” when administered to a patient. As used herein, the term a “clinically efficacious result” means a clinically useful biological response, and applies both to diagnostic and therapeutic uses. For example, a composition of the invention can be used in a method of diagnostic testing, and/or to treat, prevent and/or ameliorate symptoms of a disease or a condition in a patient.

[00047] The therapeutic agents can be any of a variety of types, including, *e.g.*, polypeptides (proteins), polynucleotides (nucleic acids), small molecules such as steroids and viral particles. The terms polypeptide and protein are used interchangeably herein, as are the terms polynucleotide and nucleic acid.

[00048] Suitable polypeptides or peptides include, but are not limited to, growth factors, cytokines, antigens, antibodies, interleukins, lymphokines, interferons, enzymes, etc., including, but not limited to, anti-IgE antibodies, tissue plasminogen activator (tPA), calcitonin, erythropoietin (EPO), factor IX, granulocyte colony stimulating factor (G-CSF), granulocyte macrophage colony stimulating factor (GM-CSF), growth hormone (particularly human growth hormone), heparin (including low molecular weight heparin), insulin, insulin-like growth factors I (IGF-I) and II (IGF-II), interleukins, interferons α , β , and γ , luteinizing hormone releasing hormone, somatostatin and analogs, vasopressin and analogs, follicle stimulating hormone, amylin, ciliary neurotrophic factor, growth hormone releasing factor, insulinotropin, macrophage colony stimulating factor (M-CSF), nerve growth factor, parathyroid hormone, α -1 antitrypsin, anti-RSV antibody, DNase, Her2, CFTR (cystic fibrosis transmembrane conductance regulator gene product, useful to treat cystic fibrosis), insulin, etc. In a preferred embodiment, the polypeptide is insulin. Polypeptides such as marker proteins can also be used.

[00049] In a preferred embodiment, the polypeptides are found within or on the surface of infectious agents, such as bacteria, viruses, protozoan or other parasites, including malaria, or the like, or as a recombinantly produced protein or polypeptide that mimics the biological

activity of a toxin produced by a bacteria. Such polypeptides can serve as immunogenic agents, for use, *e.g.*, in a vaccine.

[00050] The polypeptide can be a naturally occurring one or it can be produced recombinantly. It can be modified by any of a variety of art-recognized modifications, such as in the variant polypeptides discussed in US2002/0052475. Polypeptides used in the invention can be fragments of full-length proteins. Any desirable size (length) polypeptide can be used. For example, a peptide that comprises one or more epitopes and/or antigenic sequences can serve as an agent to elicit an immune response.

[00051] Suitable polynucleotides include, *e.g.*, vectors comprising recombinant sequences that encode therapeutic polypeptides of interest. These polynucleotides can any encode any of the therapeutic polypeptides noted above, or others. Methods to clone such sequences and to generate recombinant vectors in which the sequences of interest are operatively linked to suitable expression control sequences, are routine and conventional. Typical methods include those described in, among many other sources, Sambrook, J. *et al.* (1989) *Molecular Cloning, a Laboratory Manual*, Cold Spring Harbor Press, Cold Spring Harbor, NY, and Ausubel, F.M. *et al.* (1995). *Current Protocols in Molecular Biology*, NY, John Wiley & Sons. The phrase "expression control sequence" means a polynucleotide sequence that regulates expression of a polypeptide coded for by a polynucleotide to which it is functionally ("operably") linked. Expression can be regulated at the level of the mRNA or polypeptide. Thus, the expression control sequence includes mRNA-related elements and protein-related elements. Such elements include promoters, enhancers (viral or cellular), ribosome binding sequences, transcriptional terminators, etc. An expression control sequence is operably linked to a nucleotide coding sequence when the expression control sequence is positioned in such a manner to effect or achieve expression of the coding sequence. For example, when a promoter is operably linked 5' to a coding sequence, expression of the coding sequence is driven by the promoter. Suitable expression control sequences, such as strong constitutive or regulatable promoters, will be evident to the skilled worker.

[00052] The polynucleotide can be a naturally occurring one or it can be produced recombinantly. Polynucleotides used in the invention can be fragments of full-length nucleic acids, *e.g.*, fragments that encode fragments of full-length proteins. Any desirable size polynucleotide can be used, provided that it provides a clinically efficacious result.

[00053] Polynucleotides which can be used in compositions and methods of the invention can take any of a variety of forms that will be evident to the skilled worker, including DNA, RNA, PNA, LNA, oligonucleotides, single or double strand molecules, etc. The nucleic acids can comprise any of a number of known modifications that can aid, *e.g.*, in stabilizing them or enhancing uptake into cells. Such modifications include, *e.g.*, those discussed in USP 6,455,292.

[00054] In a preferred embodiment, the polynucleotide serves as a vaccine, *e.g.*, a DNA vaccine. The construction and use of one such DNA vaccine, which encodes the influenza haemagglutinin protein and which provides protection against at least some symptoms of influenza infection, is discussed in more detail elsewhere herein. See, *e.g.*, Examples 5-8. Such nucleic acids may encode full-length proteins or fragments thereof, *e.g.*, antigenic peptides that can elicit an immune response.

[00055] The nucleic acids can comprise coding or non-coding (*e.g.*, regulatory) sequences. In addition to encoding polypeptides, the nucleic acids can be, *e.g.*, antisense molecules or ribozymes. For a discussion of some of the well-known types of antisense molecules or ribozymes, see, *e.g.*, USP 6,455,292.

[00056] Suitable virus particles include, *e.g.*, partially or fully inactivated viral particles that can serve as antigens for vaccines, such as, *e.g.*, influenza, RSV and polioviruses. In a preferred embodiment, the virus is inactivated influenza virus. Typical strains of influenza include, *e.g.*, A/PR/8/34 and the Port Chalmers strain. Subunit vaccines, prepared by conventional methods, are also included. In another embodiment, conventional viral vectors that are suitable for intranasal administration, including but not limited to adenovirus-based vectors or AAV-based

vectors, comprising one or more genes that encode therapeutic proteins, are included. Any suitable therapeutic gene can be used, including, *e.g.*, genes suitable for treatment of cystic fibrosis.

[00057] Suitable steroids include, *e.g.*, conventional steroids for treating asthma, bronchial spasms, or other conditions, which are well known to those of skill in the art.

[00058] A therapeutic agent of interest can be initially formulated as a liquid formulation, using any of a variety of conventional liquids. Preferably, the liquid is an aqueous one, such as, *e.g.*, water (*e.g.*, injectable grade water) or any of a variety of conventional buffers, which may or may not contain salts. The pH of the buffer will generally be chosen to stabilize the protein or other type of therapeutic agent of choice, and will be ascertainable by those in the art. Generally, this will be in the range of physiological pH, although some proteins can be stable at a wider range of pHs, for example acidic pH. Thus, preferred pH ranges of the initial liquid formulation are from about 1 to about 10, with from about 3 to about 8 being particularly preferred, and from about 5 to about 7 being especially preferred. As will be appreciated by those in the art, there are a large number of suitable buffers that may be used. Suitable buffers include, but are not limited to, sodium acetate, sodium citrate, sodium succinate, ammonium bicarbonate and carbonate. Generally, buffers are used at molarities from about 1 mM to about 2 M, with from about 2 mM to about 1 M being preferred, and from about 10 mM to about 0.5 M being especially preferred, and 50 to 200 mM being particularly preferred. Generally, salts, if present in the liquid solution, are used at molarities from about 1 mM to about 2 M, with from about 2 mM to about 1 M being preferred, and from about 10 mM to about 0.5 M being especially preferred, and 50 to 200 mM being particularly preferred. Suitable salts include, but are not limited to, NaCl.

[00059] The liquid formulation can be in any of a variety of forms, *e.g.*, a solution, a suspension, a slurry or a colloid.

[00060] Optionally, the liquid formulation can comprise one or more conventional pharmaceutically acceptable excipients. "Excipients" generally refer to compounds or materials that are added to enhance the efficacy of a formulation of an API. Examples include, *e.g.*, cryoprotectants and lyoprotectants, which are added to ensure or increase the stability of the protein during the spray-freeze dry process or spray-freeze atmosphere dry process, and afterwards, for long term stability and flowability of the powder product. Suitable protectants are generally relatively free flowing particulate solids, do not thicken or polymerize upon contact with water, are essentially innocuous when inhaled by a patient or otherwise introduced into a patient, and do not significantly interact with the therapeutic agent in a manner that alters its biological activity. Suitable excipients include, but are not limited to, proteins such as human and bovine serum albumin, gelatin, immunoglobulins, carbohydrates including monosaccharides (galactose, D-mannose, sorbose, etc.), disaccharides (lactose, trehalose, sucrose, etc.), cyclodextrins, and polysaccharides (raffinose, maltodextrins, dextrans, etc.); an amino acid such as monosodium glutamate, glycine, alanine, arginine or histidine, as well as hydrophobic amino acids (tryptophan, tyrosine, leucine, phenylalanine, etc.); a methylamine such as betaine; an excipient salt such as magnesium sulfate; a polyol such as trihydric or higher sugar alcohols, *e.g.* glycerin, erythritol, glycerol, arabitol, xylitol, sorbitol, and mannitol; propylene glycol; polyethylene glycol; Pluronic; surfactants; and combinations thereof. Preferred excipients include *e.g.*, trehalose, sucrose and mannitol. Another class of excipient, mucoadhesives, are often used to increase contact of an API with mucosal surfaces. Examples of mucoadhesives include, *e.g.*, chitosan, dermatan sulfate, chondroitin, and pectin. Additionally, conventional cosolvents, which improve the solubility of APIs, can be added to liquid formulations suitable for the SFD processes disclosed herein.

[00061] Generally, when mucoadhesives are used, they are used in amounts ranging from about 1 to 95 wt %, with from about 1 to 50 wt % preferred, from about 5 to 50 wt % being especially preferred, and from about 5 to 20% being particularly preferred. In general, cryoprotectants are used at a concentration of between about 5 wt% and about 95 wt%.

[00062] In one embodiment, the dried powders of the invention are later combined with bulking agents or carriers, which are used to reduce the concentration of the therapeutic agent in the powder being delivered to a patient; that is, it may be desirable to have larger volumes of material per unit dose. Bulking agents may also be used to improve the dispersibility of the powder within a dispersion device, and/or to improve the handling characteristics of the powder. This is distinguishable from the use of bulking agents or carriers during the spray-drying process. Suitable bulking agents are generally crystalline (to avoid water absorption) and include, but are not limited to, lactose and mannitol. Accordingly, bulking agents such as lactose, if added, may be added in varying ratios, with from about 99:1 of a therapeutic agent of interest to bulking agent to about 1:99 being preferred, and from about 1:5 to about 5:1 being more preferred, and from about 1:10 to about 1:20 being especially preferred.

[00063] Liquid formulations of the invention can be atomized by any of a variety of conventional procedures. For example, the liquid can be sprayed through a two-fluid nozzle, a pressure nozzle, or a spinning disc, or atomized with an ultrasonic nebulizer or a vibrating orifice aerosol generator (VOAG). In one embodiment, a liquid formulation is atomized with a pressure nozzle such as a BD AccuSpray™ nozzle.

[00064] In a preferred embodiment, atomization conditions are optimized such that the mean mass diameter of the atomized droplets (*e.g.*, nebulized droplets) is at least about 20 μ , preferably between about 35 μ m and about 300 μ m, more preferably between about 50 μ m and about 300 μ m, still more preferably between about 50 μ m and about 100 μ m. Methods to optimize the generation of droplets of the desired size are conventional. Among the conditions that can be varied are atomization gas flow, atomization gas pressure, liquid flow rate, etc. Also, the type and size of the nozzle can be varied. Liquid drop size can be readily measured, using conventional techniques, such as laser diffraction. The size of dried particles can be measured by conventional techniques, such as, *e.g.*, scanning electron microscopy (SEM) or laser diffraction. Figures 4 and 18, *e.g.*, show typical particle size distribution of a liquid sample and a dry powder sample, respectively, as measured by laser diffraction, for samples produced by a method illustrated in Example 1.

[00065] In one embodiment, in which frozen, atomized particles are dried at about atmospheric pressure, as is discussed elsewhere herein, the size of the atomized droplets can be, *e.g.*, at least about 20 μ m, preferably between about 20 μ m and about 300 μ m, more preferably between about 35 μ m and about 100 μ m or between about 50 μ m and about 100 μ m.

[00066] Following the atomization of a liquid formulation, the droplets are rapidly frozen to form solid particles. Preferably, the droplets are frozen immediately, or substantially immediately, after the atomization step.

[00067] In one embodiment, the droplets are frozen by immersing them in a cold liquid that is below the freezing point of the liquid formulation from which the atomized droplets were formed. In a preferred embodiment, the temperature of the cold liquid is about -200° C to -80° C, more preferably between about -200° C to -100° C, most preferably about -200° C (liquid nitrogen is about -196° C). Any suitable cold liquid may be used, including liquid nitrogen, argon and hydrofluoroethers, or a compressed liquid, such as compressed fluid CO₂, helium, propane or ethane, or equivalent inert liquids, as is well known in the art. For example, in one embodiment, a liquid preparation of a therapeutic agent is atomized through a spray nozzle that is positioned above a vessel containing a suitable cold liquid, such as, *e.g.*, liquid nitrogen. The droplets freeze instantaneously upon contact with the cold liquid. Example 2 shows the preparation of a composition of inactivated flu virus particles that utilizes such a freezing procedure.

[00068] In another embodiment, the droplets are frozen by passage through a gas (*e.g.*, cold air, nitrogen, helium or argon), in a cooling chamber, wherein the gas is below the freezing point of the droplets. In a preferred embodiment, the cold gas is about -5° C to -60° C, more preferably between about -20° C to -40° C. The gas can be cooled by conventional methods, such as by cooling coils, heat exchangers or chiller condensers. The temperature of the gas can be reduced with conventional procedures, *e.g.*, with liquid nitrogen, solid carbon dioxide or an equivalent cryogenic agent to produce the subfreezing temperatures. Examples 1a and 1b

illustrate typical apparatus and methods that can be used to produce compositions of the invention, in which nebulized droplets are cooled in a gas by passage through suitable cooling chambers.

[00069] Following the formation of solid frozen particles, the particles are dried to produce a powder. By “dry” is meant having a negligible amount of liquid, *e.g.*, having a moisture content such that the particles are readily dispersible to form an aerosol, *e.g.* in an inhalation device. This moisture content is generally below about 15% by weight water, with less than about 10% being preferred and less than about 1% to about 5% being particularly preferred.

[00070] In one embodiment of the invention, the frozen droplets are dried by lyophilization (freeze-drying, under vacuum), using a conventional lyophilization apparatus. This method is generally called a “spray-freeze-dry” or SFD method, and compositions made by the method are called “spray-freeze-dry” or SFD compositions. For example, in one embodiment, when particles have been frozen by spraying them into a vessel (such as a Virtis freeze-drying flask) containing liquid nitrogen, the vessel can then be attached to a conventional lyophilizer and the excess liquid nitrogen evaporated off. The frozen aerosol is typically dried within about 48 hours and reaches a moisture level below about 1 wt%. Alternatively, droplets that have been frozen in cold air at about atmospheric pressure and, optionally, partially dried at about atmospheric pressure (as is discussed below) can then be placed in a lyophilization flask and subjected to lyophilization.

[00071] In another embodiment, the frozen droplets are dried by sublimation in a cold, desiccated gas (*e.g.*, air, nitrogen or helium) stream at about atmospheric pressure. By “about atmospheric pressure” is meant herein a pressure ranging from about one half atmosphere to about five atmospheres. The temperature of the gas can be reduced by any of a variety of conventional procedures, *e.g.*, with liquid nitrogen, solid carbon dioxide or an equivalent cryogenic agent. Particles of the invention that are dried in such a manner are sometimes referred to herein as “spray-freeze-atmosphere-dried” particles. In a preferred embodiment,

atomized droplets are frozen and dried in the same “spray-freeze-atmosphere-dry” chamber, allowing the freezing and drying procedures to be carried out in a single step.

[00072] One apparatus and method for drying solid, frozen particles in cold air at about atmospheric pressure is disclosed in Leuenberger, USP 4,608,764. See also Examples 1a and 1b herein. Other types of conventional apparatus can also be used.

[00073] In a preferred embodiment, *e.g.*, as shown in Examples 1b and 11, frozen atomized particles are dried in a cold gas at about atmospheric pressure in the presence of conditions that enhance fluidization of the particles. In a most preferred embodiment, the frozen, atomized particles are dried in the presence of vibration, internals, mechanical stirring, or combinations thereof, during the drying process. The term, “internals,” as used herein, refers to any physical barrier inside a chamber (*e.g.*, the SFD chamber) or fluidized bed, such as, *e.g.*, blades, plates or other barriers. Such treatments allow the particles to achieve a fluidized state. A method and apparatus for achieving such fluidization is discussed in Examples 1b and 11.

[00074] The method and apparatus described in Examples 1b and 11 also help to prevent channeling. Channeling is one of the most undesirable fluidization characteristics of fine particles and can occur at low or high fluidization velocity. This happens when gas passes up through voids extending from the distributor to the bed surface. These vertical channels may move across the bed with time, resulting in defluidization of the bed. There are also small cracks in the bed, which drain into these vertical channels. With increasing gas velocity, not only small channels but also large channels, also called rat-holes, are formed for some extremely cohesive particles. This difficulty arises because the interparticle forces are noticeably greater than the forces the fluid can exert on the particles.

[00075] Spray-frozen powder, which is spray-frozen by using, *e.g.*, a two-fluid nozzle, a pressure nozzle or an ultrasonic nozzle, can be very difficult to fluidize. When dried in a fluidized bed, such particles channel or agglomerate easily, making them difficult or even impossible to dry quickly and completely. The present inventors have recognized that the

introduction of vibration, internals, mechanical stirring, or a combination thereof, during the drying process, can be effective in allowing such particles to become fluidized.

[00076] In another embodiment, the frozen droplets are dried by a combination of sublimation in a cold, desiccated gas (*e.g.*, air) stream at about atmospheric pressure, as described above, and lyophilization. For example, a composition that has been partially dried at about atmospheric pressure (*e.g.*, to form a cake or a powder that still contains undesirable amounts of liquid) is removed to a lyophilizer, in which the composition is dried further.

[00077] Conventional methods can be used to collect the dried compositions. In one embodiment, the dried particles are collected on a filter, from which they can be removed for use in, *e.g.*, medical applications. See Examples 1a and 1b and Figures 1A and 1B for illustrations of such a method and an apparatus that can be employed to perform it. In another embodiment, the spray-freeze atmosphere dried particles are collected in a product vessel. Partially dried particles may form a loose cake, from which remaining moisture can be removed by further atmospheric sublimation in a cold desiccated air stream, or they can optionally be removed to a lyophilizer or other suitable device and further dried under reduced pressure (below atmospheric pressure.)

[00078] Particles dried by any of the above methods exhibit substantially the same properties (*e.g.*, particle size, porosity, and the like).

[00079] The atmospheric spray-freeze-drying process of the present invention, especially with vibration and/or internals, provides an economically feasible method of producing dried particles and increasing yield. Compositions prepared by the method can have properties that, *e.g.*, facilitate respiratory administration. Unlike the spray-freeze-drying process disclosed in, *e.g.*, United States Patent No. 6,284,282 to Maa, this embodiment of the invention produces dried particles with a single apparatus. The atomization, freezing and drying of the present invention preferably occur in a single vessel, thus eliminating the need to transfer the sample, which may result in sample contamination and reduced yield. The entire operation can also be

accomplished as a continuous operation, thus providing improved efficiency. By a “continuous operation” is meant that there is no temporal break between the steps and/or that there is no physical isolation (*e.g.*, the frozen atomized particles are not removed to a separate container for drying). Other spray-freeze-dried processes utilized for preparing pharmaceutical compositions often include a second step of lyophilization, which involves removing the frozen particles from the spray-freezing chamber and transferring the particles to a lyophilizer. Such an additional step reduces the commercial feasibility of the spray-freeze-dry process and can result in agglomeration of the particles due to partial thawing of moisture entrapped in the particles.

[00080] Following the drying procedure, a composition of the invention can achieve the form of a free-flowing powder. The dry, porous particles of the composition are roughly the same size (geometric diameter) and shape as the frozen droplets prior to drying.

[00081] Dried particles of the invention exhibit desirable aerodynamic properties. Inertial impaction and gravitational settling of dried particles determines their deposition profile in the respiratory track of an animal. Methods of determining such deposition profiles are routine and conventional in the art. For example, the aerodynamic diameter is defined as the product of the actual particle diameter multiplied by the square root of the ratio of particle density to water density. ($d_{ae} = (sg)^{1/2} d_p$, $sg = \rho_{particle} / \rho_{water}$, d_{ae} is particle aerodynamic diameter, d_p is particle diameter).

[00082] Dried powders of the invention also exhibit other desirable properties. Examples 9 and 10 show some properties of compositions of insulin made by the methods of the invention. The particles show a desirable morphology (as shown by SEM) and a desirable density. Furthermore, the particles exhibit lower amounts of residual powder remaining in a delivery device following its use, exhibit greater stability than, *e.g.*, liquid insulin formulations, and are readily reconstituted in liquid.

[00083] Compositions of the invention can be used to treat a variety of medical conditions. Among the many conditions that can be treated are diabetes, infectious diseases or any of the

conditions that can be treated by the therapeutic agents discussed elsewhere herein, or by other therapeutic agents. In a preferred embodiment, compositions of the invention are vaccines.

[00084] One aspect of the invention is a method of treating a patient in need thereof, comprising administering to said patient an effective amount of a pharmaceutical composition produced by a method of the invention. By an “effective amount” is meant herein an amount that is effective to elicit a desired response. For example, an effective amount of an immunogenic composition is an amount that is effective to elicit a detectable immune response. An effective dose can be determined empirically, according to conventional procedures, taking into account well known factors such as the age, weight, and/or clinical condition of the patient, the method of and scheduling of administration, and the like. The patient can be any animal, preferably a mammal such as, *e.g.*, a farm or other domestic animal, or a rat, mouse, hamster, guinea pig, rabbit, etc., preferably a human.

[00085] Compositions of the invention can be administered by any of a variety of routes that are known to the skilled worker, including, but not limited to, parenteral, respiratory, intranasal, intrarectal, intravaginal, sublingual, or oral routes. In one embodiment, the composition is administered to a mucosal tissue, including, but not limited to, mucosal tissue of the nasal passages and the sinuses. In a preferred embodiment, a composition of the invention is administered to a patient in need thereof via the respiratory system. By “administration through the respiratory system” or “respiratory administration” is meant herein that an agent is administered through the nose (intranasally), after which the agent passes through the nasal cavities and the sinuses and, in some cases, into the lungs.

[00086] Conventional methods of administration may be used. Suitable applicators (*e.g.*, inhalers) are known in the art. Typical delivery devices include, but are not limited to, the devices disclosed in USP 09/879,517 (filed 6/12/01) and 09/758,776 (filed 1/12/01). Dosages to be administered can be determined by conventional procedures known to those of skill in the art. See, *e.g.*, *The Pharmacological Basis of Therapeutics*, Goodman and Gilman, eds., Macmillan Publishing Co., New York. Factors to be considered include the activity of the

specific agent involved, the metabolic stability and length of action of the agent, mode and time of administration, drug combination, rate of excretion, the species being treated, and the age, body weight, general health, sex, diet and severity of the particular disease states of the host undergoing therapy. Dosages for eliciting an effective immune response (*e.g.*, dosages for effective vaccination) are well known to those of skill in the art.

[00087] Another aspect of the invention is a method of treating a disorder (*e.g.*, a condition or disease) by delivery of a therapeutic composition to a patient in need thereof, comprising administering to a mucosal membrane of the nasal and/or sinus passages of a patient a pharmaceutical composition of the invention. Another embodiment is a method of administering a therapeutic composition to a patient in need thereof, comprising administering to a mucosal membrane of the nasal and/or sinus passages of the patient a pharmaceutical composition of the invention. Another embodiment is a unit dosage receptacle or dry powder inhaler, comprising an effective amount of a pharmaceutical composition of the invention.

[00088] Compositions of the invention can achieve greater therapeutic effects following intranasal administration than do liquid formulations or other types of dry formulations, such as, *e.g.*, spray-dried formulations. See, *e.g.*, Examples 2 and 3. Therefore, the invention relates to a method of reducing the amount of a therapeutic agent that is required to produce an efficacious result following intranasal administration to a patient in need thereof, comprising administering to said patient, intranasally, an effective amount of a pharmaceutical composition of the invention.

[00089] In one aspect, the invention relates to a method to elicit an immune response in a patient, comprising administering to the patient an effective amount of an immunogenic composition of the invention. The term "immune response" as used herein encompasses, for example, mechanisms by which a multi-cellular organism produces antibodies against an antigenic material that invades the cells of the organism or the extra-cellular fluid of the organism. The antibody so produced may belong to any of the immunological classes, such as immunoglobulins A, D, E, G or M. Other types of responses, for example cellular and humoral

immunity, are also included. Immune response to antigens is well studied and widely reported. A survey of immunology is given, *e.g.*, in Roitt I., (1994). *Essential Immunology*, Blackwell Scientific Publications, London. Methods in immunology are routine and conventional (see, *e.g.*, *Currents Protocols in Immunology*; edited by John E. Coligan *et al.*, John Wiley & Sons, Inc.).

[00090] In one aspect, the invention relates to a vaccine (an agent used to stimulate the immune system of a living organism so that protection against future harm is provided). An influenza vaccine, for example, can protect a patient, at least to a finite degree, against infection by influenza. That is, the vaccine can result in the amelioration of at least some of the symptoms engendered by infection with the flu virus. A vaccine composition of the invention can take the form of, *e.g.*, protein (such as in a subunit vaccine), viral particles, or DNA that encodes an antigen of interest.

[00091] Example 2 shows the preparation of a spray-freeze-dried (SFD) composition of the invention that comprises inactivated influenza viral particles. The example shows the use of a particular strain of influenza virus. One of skill in the art will recognize that other strains of influenza can also be used.

[00092] Most infectious agents enter the body and exert their pathophysiologic effects through mucous membranes. Protection against infection can be provided at the first site of entry by neutralizing infectious agents with locally produced mucosal IgA. Although systemic immune responses are readily elicited by traditional immunization routes (such as IM, ID, etc.), mucosal responses are more difficult to achieve in general. An advantage of intranasal (IN) delivery of dry powder vaccine is its ability to elicit both systemic and mucosal immune response. In addition, IN powder vaccine delivery may reduce the required dose because of the effectiveness of mucosal powder drug intake.

[00093] Example 2 shows that intranasal delivery of a spray-freeze-dried flu vaccine, which comprises an excipient, produces equivalent antibody production as does intramuscular delivery of a much larger dose of vaccine.

[00094] Example 3 shows that SFD inactivated influenza particles can elicit IgG and IgA responses, which are enhanced when chitosan is present as an excipient.

[00095] Example 4 shows influenza stability studies. The lyophilization process does not adversely affect the stability of the particles, whereas milling of freeze-dried particles leads to dramatic decreases.

[00096] Examples 5-7 demonstrate the preparation and use of a DNA influenza vaccine. Methods of the present invention can be used to prepare and/or deliver vaccine compositions that comprise DNA molecules. Methods to engineer DNA vaccines, *e.g.*, influenza vaccines, are well known in the art, as is discussed elsewhere herein. Example 5 illustrates a model system, in which DNA plasmids encoding the marker gene, firefly luciferase, are introduced into rats intranasally, in either liquid formulations, or dry (FD) formulations prepared according to methods of the invention. Luciferase gene expression is observed in nasal, but not lung, tissue. IN administration of the powder formulation results in comparable levels of gene expression as with liquid formulation. Examples 6 and 7 illustrate how to produce a DNA vaccine comprising an influenza haemagglutinin (HA) encoding sequence, and that such a vaccine elicits a significant response when inoculated into rats. An SFD formulation containing the excipient trehalose, prepared in accordance with the present invention and administered intranasally, elicits a stronger antibody response than does a comparable amount of a liquid formulation introduced intranasally, or a formulation introduced intramuscularly.

[00097] Example 8 illustrates an immunization regimen in which, *e.g.*, priming is performed with DNA encoding influenza haemagglutinin, and a boost with influenza viral particles follows. This regimen provides unexpectedly high antibody responses.

[00098] In another aspect, the invention relates to an inventive pharmaceutical composition for respiratory administration, comprising insulin, and to methods of making the composition and using it to treat a patient. Respiratory delivery of insulin provides several advantages, *e.g.*, as compared to administration by intradermal or subdermal injection, including increased patient compliance and the elimination of the need for diabetic patients to administer frequent self injections. Some properties of insulin formulations of the invention are shown in Examples 9 and 10. In Examples 2- 10 presented herein, compositions are prepared by a spray-freeze-dry (lyophilized) method, sometimes referred to as "SFD." In the compositions of these examples, the particles have a mean average diameter of at least about 20 μ m. These compositions are comparable to compositions prepared by a spray-freeze-atmosphere-dry method (Examples 1, 11 and 12), and thus the findings also apply to compositions prepared by the latter method. In the foregoing and in the following examples, all temperatures are set forth uncorrected in degrees Celsius; and, unless otherwise indicated, all parts and percentages are by weight.

EXAMPLES

Example 1

1a) A method and apparatus for preparing spray-freeze-atmosphere-dried pharmaceutical compositions of the invention

[00099] Any of a variety of apparati can be used to produce a pharmaceutical composition of the invention. Referring to Figure 1, a spray-freeze-atmospheric-drying apparatus that can be used in accordance with the present invention is generally shown at 10. An exemplary example of the method to produce an active pharmaceutical ingredient (API), *e.g.*, one suitable for administration to the respiratory system, is as follows. A liquid feed line 12 is fluidly connected to an atomizing spray nozzle 14. A mixture of the API and a suitable liquid is disposed within the liquid feed line as will be explained further below. The spray nozzle 14 is disposed within a spray-freeze-atmospheric-drying chamber 16. In a preferred embodiment, a nebulizing line 18 (*e.g.*, using compressed gas) interfaces with the spray nozzle 14 in order to atomize the mixture. Atomizing gases may include, *e.g.*, nitrogen, oxygen, or air. Other conventional types of nozzles are also effective in generating the appropriate atomized particle size including a 2-fluid atomizer, a pressure atomizer, an ultrasonic nebulizer or even more preferably a vibrating

orifice aerosol generator (VOAG), each known to generate more uniform particle size distribution. A cold liquid (*e.g.*, nitrogen) or solid (*e.g.*, dry ice) can be placed inside the drying chamber to aid the rapid freezing of the atomized droplets.

[000100] A cooling system 20 provides cold air to the drying chamber 16 to maintain a temperature within the chamber 16 generally between about -20°C and -40°C at the primary drying stage. The temperature within the drying chamber 16 is preferably maintained well below the freezing point of the mixture. The cold air is produced by redundant cooling chambers 22 that utilize liquid nitrogen, solid CO₂, or an equivalent cooling agent to produce the subfreezing temperatures. Redundant cooling chambers 22 provide the flexibility to maintain operation of the system even if one of the chambers 22 needs to be shut down. A cold air inlet line 24 provides the atmospheric freezing air to the drying chamber 16 from the cooling system 20. A cold air return line 26 receives the cold air from the drying chamber 16 and returns the cold air to the cooling system 20 to maintain circulation between the drying chamber 16 and the cooling chambers 22. A filter 28 is disposed inside the chamber 16 preferably between the nozzle 14 and the cold air return line 26. The filter 28 collects the spray-freeze-atmospheric-dried particles of the API from which the API may be recovered for future medical use.

[000101] A temperature controller 30 is disposed between the cooling system 20 and the drying chamber 16 in the cold air inlet line 24 to maintain the temperature of the cold air injected into the drying chamber 16 in the desirable range. A supplemental air filter 32 is disposed in the cold air outlet line 26 between the chamber 16 and the cooling system 20 to collect any residual material that may escape from the chamber 16 thereby preventing the material from contaminating the cooling system 20. Valves 34 are disposed in the cold air inlet and cold air outlet lines 24, 26 between the temperature controller 30 and the supplemental filter 32 respectively in order to seal off the drying chamber 16 for maintenance or other operational procedures.

[000102] A pump or blower 36 is disposed between the supplemental air filter 32 and the cooling system 20 in the cold air outlet line 26 to circulate cooling air through the cooling system 20 and a chamber 16. Inlet and outlet valves 38 are disposed at an inlet 40 and outlet 42 of each cooling chamber 22 enabling each cooling chamber 22 to be separately sealed off from the cold air circulation line 24, 26 for maintenance or other operational procedures. Additionally, a bypass valve 44 is disposed in a bypass valve line 46 fluidly connected between the cold air inlet line 24 and the cold air outlet line 26 in order to allow for circulation of the cooling air through the cooling system 20 when the chamber 16 is sealed off from the cooling system 20.

[000103] Because the spray nozzle 14 is disposed within the chamber 16, the nozzle's 14 operational temperature is below the freezing point of the mixture being provided to the spray nozzle 14. Therefore, the spray valve 14 has a tendency to freeze preventing the mixture from being atomized inside the chamber 16 in an appropriate manner. Thus, heating tape 48 is operatively connected to the valve 14 to maintain the valve at a temperature above the freezing point of the mixture. Other conventional methods of maintaining the spray nozzle 14 at a temperature above the freezing point of the mixture may be used as would be known to those of skill in the art.

[000104] During operation, the atomized API is introduced to the chamber 16 through the spray nozzle and is rapidly frozen by the cold air also being introduced to the chamber 16 from the cooling system 20. The air circulating within the chamber 16 will preferably maintain the particles in a fluidized state. While the particles are maintained in the fluidized state and, additionally, are collected in the filter 28, the circulating cooling air will dry the particles by removing the liquid that may be entrapped in the now solid particle, that have been frozen in the chamber 16. Continued circulation of the cooling air will reduce the moisture in each of the particles to a negligible amount. As primary drying is completed, the circulating gas can optionally gradually be increased to room temperature to facilitate secondary drying and reduced condensation during sample removal.

[000105] Preferably, the spray nozzle 14 will direct the atomized mixture toward the inlet cooling line 24 inside the chamber 16. However, it should be understood that the spray nozzle 14 may be directed toward the outlet cooling line 26 or any other side of the drying chamber 16 necessary to optimize the spray freeze atmospheric drying process. Further, the spray nozzle 14 may be selected to produce various atomized particle sizes as may be desired for any given API and delivery method.

[000106] Alternatively, the frozen and dried particles are optionally removed from the filter 28 and introduced to a lyophilizer or other suitable device, which operates at a reduced atmospheric pressure, wherein residual moisture is removed and the particles are thoroughly dried. The lyophilizer dehydrates the particles while the particles are maintained in a frozen state as the water passes from the solid phase directly to the vapor phase, as is known in the art.

[000107] Inclusion of a spray step in the spray-freeze-atmospheric-drying process allows for the processing of the solution to a dried particulate matter at about atmospheric pressure. The processing of the spray frozen substances at about atmospheric pressure provides for APIs that are readily aerosolized, and that are suitable for administration to a patient. In addition, the method as taught by the present invention provides an economical means of commercialization for producing suitable API's.

1b) A method and apparatus for preparing spray-freeze-atmosphere-dried pharmaceutical compositions of the invention, using a vibrational fluidized bed with internals

[000108] A variant of the apparatus shown in Fig. 1A is shown in Fig. 1B. This variant comprises means for vibration (49) as well as special internals (50). The vibration and internals allow the solid, frozen particles to achieve a fluidized state as they are dried by sublimation in a cold desiccated air stream at about atmospheric pressure. This is especially useful when the frozen particles are sticky or cohesive, and is valuable in the powder cake building process when a sticky frozen powder is fluidized and elutriated.

[000109] To increase the product yield, a completely sealed system may be designed to keep frozen powder from escaping. A filter disc or paper filter disk or paper filter may be used to trap the powder elutriated from the fluidized bed below. Vibration, intervals, mechanical stirring, or combinations thereof are useful when a sticky frozen powder is fluidized and elutriated. As the sublimation proceeds, the frozen particles become porous (lighter) and the aerodynamic behavior changes. The partially dried particles may form a loose cake on exit at the disk filter, from which the remaining moisture may be removed, *e.g.*, by sublimation at about atmospheric pressure, using a cold desiccated gas stream.

[000110] Similar to the method in Example 1a, the atomized API is introduced to the chamber 16 through the spray nozzle and is rapidly frozen by the cold air also being introduced to the chamber 16 from the cooling system 24 (see Fig.1B). During operation, the vibrator 49 is turned on in an optimized frequency (0-100Hz) and amplitude. The cold, dried fluidizing gas from cooling line 24 entering from the bottom of the chamber 16 fluidizes the frozen particles. Large particle agglomerates are broken under vibration as well as the assistance of special internals (static blades) 50 located inside chamber 16. Channeling normally occurring with cohesive powder is reduced or completely eliminated in such an operating condition. In most cases small frozen particles are easily elutriated and carried out by the fluidizing gas to the filter 28. A powder cake on the filter 28 is gradually built up in the fluidization and elutriation process.

[000111] A high flow-rate is available because of the use of a particle sealed system such as described and is recommended in the powder cake building process to increase the drying rate. A high flow-rate is also used in the drying process because the drying rate of frozen powder is much faster in a fixed bed state at high flow-rate than that in a slow fluidized bed state. The atmospheric spray-freeze-drying of the present invention with vibration and/or internals provides an economically feasible method of producing dried particles and increasing the yield.

[000112] Alternatively, a fast or circulating fluidized bed may be used in such a drying process if a higher drying rate is expected. A fast or circulating fluidized bed normally consists of a dense fluidized bed at the bottom and a dilute fluidized bed at the top as well as a powder returning system. In such a process, frozen particles are fluidized and carried by cold fluidizing air and collected by a cyclone at the top then returned to the dense fluidized bed through a specially designed powder valve. For easier cooling, an internal cyclone is preferred and the returned powder valve, which is specially designed with certain resistance, will only allow powder to come down rather than fluidizing air by pass. In order to break up the particle agglomerates and improve particle circulation, similar internals are also preferably used.

[000113] Alternatively, the frozen and dried particles are optionally removed from the filter and introduced to a lyophilizer or other suitable device, which operates at a reduced atmospheric pressure, wherein residual moisture is removed and the particles are thoroughly dried. The lyophilizer dehydrates the particles while the particles are maintained in a frozen state as the moisture passes from the solid phase directly to the vapor phase, as is known in the art.

[000114] The atmospheric spray-freeze-drying of the present invention with vibration and/or internals provides an economically feasible method of producing dried particles and increasing the yield.

Example 2 – Nasal delivery of inactivated influenza virus particles

[000115] Dry powder formulations of whole, inactivated, influenza virus A/PR/9/34 H1N1 particles were prepared in a spray-freeze dried batch process. A flu virus preparation was mixed into an aqueous solution, then atomized with a BD AccuSpray™ nozzle. Liquid particle size data were obtained with a Sympatech diffractometer measuring at approximately 2 inches from the nozzle tip. The median diameter of particles produced at these concentrations was approximately 50 microns. A typical particle size distribution produced by the BD AccuSpray™ nozzle is shown in Fig. 4. Liquid nitrogen was placed in a Virtis freeze-drying flask and the flask was positioned beneath the spray nozzle. The distance between the nozzle and liquid

nitrogen was about three inches. The nebulized liquid droplets froze instantaneously upon contact with the liquid nitrogen. The flask was attached to a lyophilizer and immediately the excess liquid nitrogen was evaporated off. The frozen aerosols were typically dried within 48 hours and reached a moisture level below about 1 wt%.

[000116] In one experiment, testing was conducted to determine the strength of the immune response following intranasal (IN) or intramuscular (IM) delivery of various formulations of influenza vaccine. The study was conducted on rats and the following groups were evaluated:

Group 1 – IN. 100ug of influenza* Ag in 50 µl volume of liquid.

Group 2 – IM injection. 100µg of influenza* Ag in 50 µl volume of liquid

Group 3 – IN delivery. 100µg influenza* Ag in 10mg trehalose, freeze-dried powder

Group 4 – IN delivery. 100µg influenza* Ag in 10mg trehalose+chitosan, freeze-dried powder

Group 5 – IN delivery. 100µg influenza* Ag in 10mg trehalose+chitosan, spray-freeze-dried (SFD) powder

Group 6 – IN delivery. 10mg freeze-dried trehalose only

*Inactivated whole Influenza virus A/PR/8/34 H1N1

[000117] Rats were immunized three times, at week 0, week 3, and week 6. Serum samples were collected at week 3, week 5 and week 8 and nasal lavage fluid was collected at week 8.

[000118] Subsequent to each delivery of flu vaccine, blood samples were taken to determine the magnitude of the immune response to the flu vaccine as measured by the antibody (Ab) response to the vaccine. Also determined was the amount of powder that was delivered during each vaccination. Conventional methods were used to determine factors such as the potency of immune response and the amount of powder delivered. Figure 2 shows the serum Ab titers following each immunization. In summary, even though the serum Ab titers following IM delivery were cumulatively higher than those delivered by the intranasal deliveries, IN delivery of a spray freeze-dried flu vaccine was able to reach comparable levels of serum Ab response, in spite of low vaccine dose delivery (as low as 0-10% at the second immunization). In summary,

this experiment suggests that with the full vaccine dose, IN delivery of spray freeze-dried flu vaccine is able to elicit comparable levels of serum Ab response as that of the IM group, and better responses than that of IN liquid group.

As shown in Table 1, all IN flu vaccination groups are able to elicit positive nasal IgA response as contrast to negative nasal IgA titers following IM injection and an IN negative control. This study demonstrates that IN delivery of SFD flu vaccine is able to elicit both nasal mucosal responses and systemic immune responses.

Group 1 (IN, liquid)	Group 2 IM	Group 3 IN, powder	Group 4 IN, chitosan	Group 6 SFD, chitosan	Group 7 IN Trehalose
80	<20	<20	20	40	<20
160	<20	80	20	40	<20
160	<20	<20	40	40	<20
160	<20	20	40		

Table 1 - Nasal IgA titers

Example 3 - Comparison of Serum and Nasal Mucosal Immune Responses Following IN Delivery of SFD Flu Vaccine with/without Chitosan

[000119] This dose-ranging example compares immune responses of Brown Norway rats following IN delivery of SFD flu whole virus with and without chitosan. The following groups, each containing 4 rats, were evaluated:

- 1 – IN. 1µg of flu* Ag in 5mg trehalose, SFD
- 2 – IN. 1µg of flu* Ag in 5mg trehalose+ chitosan, SFD
- 3 – IN. 10µg of flu* Ag in 5mg trehalose, SFD
- 4 – IN. 10µg of flu* Ag in 5mg trehalose+ chitosan, SFD

Inactivated whole Influenza virus strain A/PR/8/34 H1N1 was used in this example. The rats were immunized IN three times. The results, as shown in Figures 13A and 13B, indicate that a 10µg dose of flu vaccine elicits stronger serum Ig and nasal IgA responses than does a dose of 1µg, and that formulations with chitosan elicit a serum Ab response that is stronger than formulations without chitosan.

Example 4 – Influenza Activity Studies

[000120] A series of experiments and milling processes affect influenza (flu) vaccine activity. A haemagglutinin assay (HA) was adopted in the studies as an indicator of influenza activity. This assay tests HA titers of influenza vaccine based on the ability of influenza virus to haemagglutinate chicken red blood cells, an indicator of influenza vaccine potency. Briefly, two powder samples of inactivated influenza virus particles were prepared; the first was lyophilized, the second was lyophilized then milled. The sample was milled using a Wig-L-Bug ball micromill. This mill uses a one inch stainless steel vial with an endcap. The sample is placed into the vial along with a single stainless steel ball bearing, capped, and secured into position on the mill. The vial is vibrated from end to end at a rate that is variable for a prescribed time period. After milling, the sample is removed using a small spatula. Lyophilized and milled influenza powders were reconstituted back to original liquid influenza vaccine concentrations based on total protein concentration. The activity of reconstituted influenza vaccines was determined by comparing their HA titer to that of original influenza vaccine. The results are shown below:

Original liquid flu vaccine	HA titer>60 billion
Lyophilized flu vaccine	HA titer>60 billion
Lyophilized and milled flu vaccine	HA titer = 4352
Original liquid flu vaccine	HA titer>60 billion
Spray-freeze-dried flu vaccine	HA titer>60 billion

[000121] These data indicate that the lyophilization process doesn't substantially affect vaccine activity in terms of HA titer. However, the milling process decreased HA titer dramatically. The same HA titer study was used to evaluate whether the SFD method would affect the activity of influenza vaccine. Influenza powder made by the SFD method was reconstituted back to original liquid influenza vaccine concentration based on total protein concentration. The activity of reconstituted influenza vaccines was determined by comparing their HA titer to that of original influenza vaccine.

[000122] The result demonstrates that the SFD method did not detrimentally affect influenza vaccine activity and that free-flowing influenza vaccine powder can be prepared, ready for administering, with full preservation of vaccine activity and without additional milling operation.

Example 5 – IN Delivery of Plasmid DNA (“Naked DNA”) Encoding Luciferase

[000123] Effective gene therapy and DNA-based immunization requires protein expression from the delivered gene. As such, reporter gene systems are commonly used as preliminary models for determining the feasibility of such therapies. This study evaluated luciferase activity following IN delivery of various DNA doses and formulations.

A. Liquid Formulation

[000124] A plasmid was used in which firefly luciferase-encoding sequences are placed under the control of a CMV promoter (pCMV-LUC). pCMV-LUC was obtained from Aldevron LLC, located at 3233 15th St. South, Fargo, ND, 58104. A liquid formulation was prepared and doses of 50 µg or 100 µg in a volume of 50 µl in PBS were delivered IN to Brown Norway rats. Nasal and lung tissues were collected 24 hours after DNA delivery, homogenized and tested for luciferase activity using a luminescence assay.

[000125] Fig. 5 shows that luciferase activity was detected in nasal tissue, but not in lung tissue. IN delivery of 100 µg DNA resulted in higher luciferase activity than 50 µg of DNA.

B. Comparison of Liquid and Dry Powder Formulations

[000126] Liquid formulations of pCMV-LUC were prepared as described above. Dry powder (FD) formulations were prepared by lyophilization and milling as described in Example 4, using trehalose as an excipient. In some preparations, the excipient chitosan was also present. Doses of 100 µg in 50 µl of PBS of the liquid formulation, or 100 µg in 5 mg total powder for the powder formulations were administered to rats and samples were analyzed as above.

[000127] Fig. 6 shows that both dry powder and liquid formulations result in high luciferase activity in nasal tissue, but not in lung tissue. IN delivery of dry powder results in comparable levels of luciferase activity as that obtained with the liquid formulation. This result indicates the feasibility of delivering DNA in the form of SFD powders.

Example 6 – A DNA Influenza Vaccine

[000128] A plasmid was prepared, using conventional recombinant techniques, in which a DNA sequence encoding the influenza virus surface antigen haemagglutinin was placed under the control of CMVPro sequences of a CMV early promoter (Robinson *et al.* (1995) in *Vaccines 95*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 69-75). The plasmid (pFLU-HA) was purified by conventional techniques and inoculated into rats by several methods: intra-muscular (IM), intranasal, liquid formulation (IN-liquid) and intranasal-SFD, with the excipient trehalose (IN-SFD-trehalose). The serum Ab response was measured. As shown in Fig. 3, IN delivery of SFD flu vaccine elicits serum Ab responses at least comparable to that of IM injection and higher responses than that of IN liquid delivery. IN SFD flu vaccination has shown its advantages over conventional IM injection in this study.

Example 7 – Immunization with DNA Influenza Vaccines

[000129] Based on the preliminary result from IN DNA powder delivery, as shown above, a larger scale study was conducted to compare immune response following various formulations of DNA vaccine. The pFLU-HA plasmid was prepared in a liquid formulation (in PBS) or in a dry powder formulation that optionally contained trehalose/chitosan. Two types of dry formulations were used: FD (standard freeze-dry formulation) and SFD (spray-freeze dry (lyophilized) formulation). Doses of 50 µg of plasmid DNA of each dry formulation were administered intranasally (IN), and a comparable dose of the liquid formulation was administered intramuscularly (IM) or IN to Brown Norway rats on days 0, 21 and 42. Serum samples were taken on days 21, 35 and 56, and nasal lavage was taken on day 56.

[000130] Figure 7 shows that serum antibody titers following IN administration of the powder formulations were comparable to IM injection, and stronger than IN administration of the liquid formulation. The IN SFD/chitosan formulation elicits the strongest Ab response at the early stage of immunization. This suggests that an SFD/chitosan administration may allow for reduced vaccination doses and/or frequency of administration.

[000131] Table 2 shows that lack of nasal IgA responses in all experimental groups except a few animals from groups with IN SFD DNA delivery.

	IM	IN liquid	FD	FD/chtsan	SFD	SFD/chtsn
Rat 1	<10	<10	<10	<10	<10	<10
Rat 2	<10	<10	<10	<10	<10	<10
Rat 3	<10	<10	<10	<10	<10	<10
Rat 4	<10	<10	<10	<10	<10	<10
Rat 5	<10	<10	<10	<10	10	10
Rat 6	<10	<10	<10	<10	<10	10
Rat 7	<10	<10	<10	<10	<10	<10
Rat 8	<10	<10	<10	<10	<10	<10
Rat 9			<10	<10	<10	<10

Table 2 - Nasal IgA titers

[000132] The overall result from this study shows potential advantages of SFD/chitosan DNA formulation in eliciting better serum and nasal mucosal immune response in animal models.

Example 8 – Flu DNA Primary plus Viral Boost Regimen

[000133] A recently developed vaccination approach for numerous diseases, including HIV, is the so-called “prime-boost” approach, wherein the initial “priming” immunization and secondary “boosters” employ different vaccine classes (*Immunology Today* Apr 21(4), 163-165, 2000). For example, one may prime with a plasmid DNA version of the vaccine followed by a subsequent boost with a subunit protein, inactivated virus or vectored DNA preparation. This strategy was adopted in this study to investigate the potency of immune response when various formulations are delivered via different immunization routes and different combinations of immunization route.

[000134] pFLU-HA plasmid was prepared in liquid or dry formulations (FD, with and without trehalose/chitosan, and SFD, with and without trehalose/chitosan) as described in Example 5. These formulations were used for immunizations number 1 and 2 (primary immunizations).

[000135] For immunization number 3 (boost immunization), inactivated influenza was prepared in PBS (liquid formulation) or in trehalose/chitosan (dry powder formulation). Each group was divided into 2 subgroups: one received IM liquid influenza virus immunization and the other received IN influenza virus immunization of various formulations.

[000136] Brown Norway rats were immunized with selected formulations on days 0, 21 and 42. Serum samples were taken on days 21, 35 and 56, and nasal lavage, vaginal lavage and BAL were taken on day 56.

[000137] A summary of the vaccination regimens and the Ab responses observed is shown in Tables 3A through 3G. These data show that:

- DNA priming + influenza virus boost elicits a much stronger serum anti-influenza Ab titers than DNA or virus alone
- IN SFD/chitosan DNA priming +IM virus boost elicits a much stronger nasal IgA response than DNA or IM virus alone.
- IN SFD/chitosan DNA priming +IM virus boost elicits a stronger nasal IgA response than IN FD/chitosan DNA priming +IM virus boost.
- IN SFD/chitosan DNA priming +IM virus boost elicits a stronger nasal IgA response than the same strategy without chitosan
- IN SFD/chitosan DNA priming +IM virus boost elicits a stronger nasal IgA response than IN liquid DNA priming +IM virus boost
- Positive IgA and total Ig titers were detected in BAL fluid of all groups

[000138] Table 3 (below) shows a trial with flu DNA priming followed by a viral boost. Table 3A shows flu DNA priming and boost trial: 1st bleed serum total Ig titers (day 21). Table 3B shows flu DNA priming and boost trial: 2nd bleed serum Ig titers (day 35). Table 3C shows

flu DNA priming and boost trial: 3rd serum total Ig titers (day 56). Table 3D shows flu priming and boost trial: Nasal IgA titers (day 56). Table 3E shows flu priming and boost trial: Vaginal IgA titers (day 56). Table 3F shows flu priming and boost trial: BAL IgA titers (day 56). Table 3G shows flu priming and boost trial: BAL total Ig titers (day 56).

[000139] As shown in Table 3A to 3C, powder groups in this study generally elicit stronger serum Ab titers than liquid groups. In addition, the strong nasal IgA responses following IN SFD/chitosan priming plus an IM boost (Table 3D, group 5a) are unexpected, given that neither DNA immunization alone, nor IM virus immunization alone, elicits a positive nasal IgA response. IN SFD DNA/chitosan primary plus an IM boost elicits a better immune response than IN DNA or IM virus alone.

Table 3A

Group ID	Group 1	Group 2	Group 3	Group 4	Group 5
Immunization 1 (DNA)	IN Liquid	IN FD	IN SFD	IN FD/ chitosan	IN SFD Chitosan
Rat 1	<50	6400	51200	6400	6400
Rat 2	<50	12800	12800	3200	25600
Rat 3	1600	6400	25600	6400	6400
Rat 4	400	6400	12800	25600	25600
Rat 5	<50	800	6400	25600	3200
Rat 6	<50	25600	6400	12800	3200
Rat 7	800	25600	12800	12800	12800
Rat 8	<50	12800	12800	6400	25600
Rat 9	200	12800	12800	3200	25600
Rat 10	<50	12800	12800	12800	3200
Avg. titers	300	12240	16640	11520	13760

Table 3B

Group ID	Group 1	Group 2	Group 3	Group 4	Group 5
Immunization 1+2 (DNA)	IN Liquid	IN FD	IN SFD	IN FD/ chitosan	IN SFD chitosan
Rat 1	400	12800	102400	dead	12800
Rat 2	400	51200	12800	12800	25600
Rat 3	1600	6400	25600	51200	12800
Rat 4	<50	25600	25600	51200	51200
Rat 5	200	1600	25600	6400	3200
Rat 6	50	25600	51200	51200	dead
Rat 7	3200	12800	102400	12800	25600
Rat 8	<50	25600	12800	dead	dead
Rat 9	1600	25600	dead	25600	dead
Rat 10	200	25600	6400	102400	25600
Avg. titers	765	21280	40533	39200	22400

Table 3C

Group ID	Group 1A	Group 1B	Group 2A	Group 2B	Group 3A	Group 3B	Group 4A	Group 4B	Group 5A	Group 5B
Immunization 1+2 (DNA)	IN Liquid	IN liquid	IN FD	IN FD	IN SFD	IN SFD	IN FD/ chitosan	IN FD/ chitosan	IN SFD chitosan	IN SFD/ chitosan
Immunization 3 (virus)	IM	IN liquid	IM	IN FD	IM	IN SFD	IM	IN FD/ chitosan	IM	IN SFD/ chitosan
Rat 1	51200	25600	409600	819200	819200	819200	dead	409600	1638400	dead
Rat 2	25600	102400	819200	819200	819200	1638400	1638400	409600	1638400	204800
Rat 3	819200	25600	819200	819200	819200	dead	3276800	dead	1638400	dead
Rat 4		51200	409600	409600	819200	409600	3276800	819200	1638400	dead
Rat 5	102400	12800	409600	819200	819200		819200	819200	819200	102400
Rat 6					819200					
Avg. titers	249600	43520	573440	737280	819200	955733	2252800	614400	1474560	153600

Table 3D

Group ID	Group 1A	Group 1B	Group 2A	Group 2B	Group 3A	Group 3B	Group 4A	Group 4B	Group 5A	Group 5B
Immunization 1+2 (DNA)	IN liq.	IN liq	IN FD	IN FD	IN SFD	IN SFD	IN FD/chtsn	IN FD/chtsn	IN SFD/chtsn	IN SFD/chtsn
Immunization 3 (virus)	IM	IN liq.	IM	IN FD	IM	IN SFD	IM	IN FD/chtsn	IM	IN SFD/chtsn
Rat 1	<10	20	<10	40	<10	160	dead	40	10	dead
Rat 2	<10	40	<10	40	<10	40	<10	10	40	40
Rat 3	<10	40	<10	20	<10	dead	<10	dead	40	dead
Rat 4	<10	20	<10	40	<10	40	<10	40	20	dead
Rat 5	<10	<10	<10	40	<10		<10	20	<10	80
Rat 6					<10					
Avg. titers	<10	24	<10	36	<10	80	<10	27.5	22	60

Table 3E

Group ID	Group 1A	Group 1B	Group 2A	Group 2B	Group 3A	Group 3B	Group 4A	Group 4B	Group 5A	Group 5B
Immunization 1+2 (DNA)	IN liq.	IN liq.	IN FD	IN FD	IN SFD	IN SFD	IN FD/chtsn	IN FD/chtsn	IN SFD/chtsn	IN SFD/chtsn
Immunization 3 (virus)	IM	IN liq.	IM	IN FD	IM	IN SFD	IM	IN FD/chtsn	IM	IN SFD/chtsn
Rat 1	<8	<8	<8	<8	16	<8	dead	<8	<8	dead
Rat 2	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8
Rat 3	<8	<8	<8	<8	<8	dead	<8	dead	<8	dead
Rat 4	<8	<8	<8	<8	<8	<8	<8	<8	<8	dead
Rat 5	<8	<8	<8	<8	<8		<8	<8	<8	<8
Rat 6					<8					

Table 3F

Group ID	Group 1A	Group 1B	Group 2A	Group 2B	Group 3A	Group 3B	Group 4A	Group 4B	Group 5A	Group 5B
Immunization 1+2 (DNA)	IN liq.	IN liq.	IN FD	IN FD	IN SFD	IN SFD	IN FD/chtsn	IN FD/chtsn	IN SFD/chtsn	IN SFD/chtsn
Immunization 3 (virus)	IM	IN liq.	IM	IN FD	IM	IN SFD	IM	IN FD/chtsn	IM	IN SFD/chtsn
Rat 1	4	4	4	16	64	32	dead	16	16	dead
Rat 2	<1	4	4	4	4	16	4	<1	32	8
Rat 3	2	32	<1	4	4	dead	2	Dead	4	dead
Rat 4	<1	16	<1	4	<1	16	16	16	16	dead
Rat 5	2	4	2	2	2		4	<1	4	8
Rat 6					32					

Table 3G

Group ID	Group 1A	Group 1B	Group 2A	Group 2B	Group 3A	Group 3B	Group 4A	Group 4B	Group 5A	Group 5B
Immunization 1+2 (DNA)	IN liq.	IN liq.	IN FD	IN FD	IN SFD	IN SFD	IN FD/chtsn	IN FD/chtsn	IN SFD/chtsn	IN SFD/chtsn
Immunization 3 (virus)	IM	IN liq.	IM	IN FD	IM	IN SFD	IM	IN FD/chtsn	IM	IN SFD/chtsn
Rat 1	64	32	256	256	4096	128	Dead	128	512	Dead
Rat 2	32	128	256	256	2048	1024	256	32	1024	256
Rat 3	256	32	256	512	256		256	Dead	128	Dead
Rat 4	8	64	256	128	512	512	1024	128	1024	Dead
Rat 5	64	8	64	256	128		512	256	512	32
Rat 6					1024					
Avg. titers	85	53	218	282	1344	555	512	136	640	144

Example 9 – Insulin and Insulin with an Excipient by SD and SFD

[000140] Summarized in Table 4 are experimentally determined physical characteristics of spray-dried and spray freeze-dried insulin compositions in combination with a lactose excipient. During the formation of these compositions the outlet temperature of the solution being sprayed from the atomizer was also monitored along with percent yield and tap density (g/cm^3) of the particles. The samples and tabulated data are indicated below:

Table 4

Solution	Process	Tap Density (grams per centimeter cubed)
Pure Insulin	Spray dried	.29
40/60: Insulin/ Lactose	Spray dried	.49
Pure Insulin	Spray freeze dried (SFD)	0.01
40/60: Insulin/ Lactose	Spray freeze dried(SFD)	0.06

As is depicted in the table, the tap densities of spray freeze-dried essentially pure insulin and the 40/60 solution of insulin/lactose were significantly lower than those of spray-dried insulin and insulin/lactose particles. The SFD powder compositions possessed better retention of protein stability and bioactivity than spray dried compositions. In contrast, spray-dried pure insulin and insulin/lactose (40/60) powders were significantly denser, having tap densities of 0.29 and 0.49 g/cm^3 . Although not wishing to be bound by any particular theory, applicants believe that these advantages ensue because spray-freeze-drying takes place at a much lower temperature (roughly 100 °C lower) than conventional spray-drying.

[000141] A 2% by weight solution of insulin in water and a 5% by weight of insulin/lactose (40/60) were processed by a spray freeze-dry (lyophilization) procedure of the invention. The

resulting porous particles exhibited aerosol characteristics suitable for delivery into the respiratory system of an animal.

[000142] Applicants' testing has indicated that the smallest aerodynamic particle size obtained with the spray freeze drying method consisted of excipient-free insulin, *i.e.*, essentially pure insulin. However, it is understood to be within the scope of the invention that formulations consisting of insulin and excipient are suitable for the intended purpose of administering to the respiratory system. In addition, the SFD insulin particles (Fig. 19) showed virtually no detectable hydration when exposed to ambient conditions (20°C, 53% RH) for 15 minutes and during storage compared to spray-dried insulin/lactose particles. In contrast, Figure 20 shows the retained morphology of the SFD insulin/lactose particles after exposure to the same ambient conditions for 15 minutes. Typically, SFD insulin/lactose particles pick up at least 2 to 5wt% moisture upon exposure to relative humidity >50%. These results indicate that SFD porous insulin produced by the methods of the invention possess relatively high resistance to ambient moisture compared to SFD porous insulin/lactose.

[000143] Another desired attribute provided for by the methods taught in the instant invention is the substantial reduction of residual powder remaining in the delivery device after use. The percent admitted dose from a particular delivery device is calculated gravimetrically. By way of example, API's prepared in accordance with the methods of the instant invention when used in conjunction with delivery devices such as those disclosed in United States Patent Application Nos. 09/879,517 filed June 12, 2001 and 09/758,776, filed January 12, 2001 resulted in less than 5% residual powder remaining in the delivery device. By comparison, approximately 20 wt% of spray dried powder remained in the same delivery device. In addition, aerosolization of the SFD powders was visually observed to be efficient and left essentially no residual powder in the respiratory delivery devices. Photographs of drug carrier capsules from this device with SD (lower left capsule) and SFD (upper right capsule) powders after rupturing the capsule membranes are shown in Figure 21, which clearly demonstrates excellent aerosol properties of SFD powders.

Example 10 – Stability of SFD Insulin Powder vs. Liquid Insulin

[000144] The stability of the SFD insulin was evaluated relative to U500 Liquid Lilly Humulin - R with m-cresol, a standard liquid insulin widely being administered today. SFD pure insulin and insulin/trehalose were tested for eight weeks at 40 °C and 75% relative humidity. Pure insulin was also tested while sealed in an aluminum overwrap. The liquid insulin was tested at 25 °C (room temperature) and 60% relative humidity. The percentage of Desamido formation relative to the sample was measured to determine the stability of each sample. The percent formation of Desamido was determined initially, at one week, at two weeks, at four weeks, at six weeks, and at eight weeks.

[000145] Desamido is known to adversely affect diabetic patients by producing an immunity in the patient to insulin. The FDA has issued limits to the amount of desamido content in insulin to less than 10%.

[000146] The SFD pure insulin proved to be exceptionally stable throughout the eight week evaluation. The results for SFD pure insulin were:

	<u>Initial</u>	<u>1 week</u>	<u>2 weeks</u>	<u>4 weeks</u>	<u>6 weeks</u>	<u>8 weeks</u>
%Desamido	0	0.329	0	0.222	1.015	2.66

[000147] The SFD pure insulin sample that was wrapped with aluminum exhibited chemical stability throughout the eight week trial period. Until the six week evaluation, the amount of desamido detected was below the statistical error and was considered negligible. The amount of desamido detected peaked at the six week evaluation at 0.75%. The results for SFD pure insulin with the aluminum overwrap were as follows:

	<u>Initial</u>	<u>1 week</u>	<u>2 weeks</u>	<u>4 weeks</u>	<u>6 weeks</u>	<u>8 weeks</u>
%Desamido	0	0.396	0.12	0	1.752	0.750

[000148] SFD insulin was evaluated with Trehalose excipient. In this case, no desamido was detected until week 6. Overall, the addition of Trehalose significantly improved the shelf life of the SFD insulin. The results indicate:

	Initial	1 week	2 weeks	4 weeks	6 weeks	8 weeks
%Desamido	0	0	0	0	0.24	1.57

[000149] The baseline liquid insulin was also evaluated over 8 weeks to determine the amount of desamido growth. The liquid insulin proved to be much less stable relative to the SFD insulin over the eight week evaluation period. In addition to the weekly testing conducted, the liquid insulin was also evaluated at 24 and 72 hours. No desamido growth was detected at these early evaluations. A significant variable to the study is that the liquid insulin was evaluated at much less severe storage conditions than was the SFD insulin. Additionally, the liquid insulin included the chemical preservative m-cresol to slow the growth of the desamido.

	Initial	1 week	2 weeks	4 weeks	6 weeks	8 weeks
%Desamido	0	1.216	1.23	1.32	1.640	2.13

[000150] Each of the SFD insulin samples proved to be significantly more stable than the liquid insulin. The liquid insulin degraded significantly faster than the SFD powder formulation. The SFD insulin with the aluminum overwrap proved to be the most stable sample tested through the eight week trial at 40°C at 75% relative humidity. The results indicate that the SFD powder insulin is much more stable than presently commercially available liquid insulin formulations.

[000151] Figure 10 shows the growth rate of desamido of the SFD pure insulin tested against the liquid insulin.

Example 11 – Vibrational Fluidized-Bed Spray Freeze Drying

[000152] Spray-freeze-dried, porous particles were successfully produced at about atmospheric pressure (no vacuum) in the presence of intervals and vibration, as described elsewhere herein. The internals and vibration-assisted fluidization were shown to enhance the sublimation of

frozen aerosol. A 20 wt% mannitol solution (Fig. 11) (i.e. 80wt% moisture) was dried at -20°C in less than 40 minute to reach at 0.3 wt%. This is superior to, *e.g.*, the results obtained by Leuenberger (USP 4,608,764).

[000153] The moisture content and drying time of 20 wt% mannitol (as a function of drying gas velocity) is shown in Figure 11. The lowest drying curve presented in the table was generated in BDT where the rest of the data were extracted from the literature (Leuenberger, USP 4,608,764). Based on the result from TGA, the residual moisture was about 0.3% after 40-minute drying at gas velocity of 2 m/s. The morphology of the particles is shown to be porous as indicated by the SEM as shown in Figure 12. The spray frozen aerosol below $20\text{ }\mu\text{m}$ exhibited strong cohesive force between particles and the fluidization gas could simply channel upward through the bed. When the spray frozen powder was dried at 0.39 m/s, -20°C without internal and vibration, it was found that 95 wt% of powder remained at the bottom of the bed due to channeling after drying for three hours. The channeling in the fluidized-bed resulted poor drying efficiency. Roughly 5wt% of powder elutriated to the top (after drying for three hours) of the bed and was dried at much faster rate than the powder collected from the bottom of the bed. Elutriation is the process by which fine particles are carried out by fluidizing gas from a fluidized bed when gas superficial velocity is higher than particle/agglomerate terminal velocity. The effect of drying gas velocity and elutriation in a fluidized-bed is explained in Example 13 from mass transfer analysis. The moisture levels of the samples collected from top and bottom at various time points are shown in Figure 14. Only 5% of spray frozen powders elutriated to the top of the drying bed. 95% of frozen powder remained at the bottom; the poor drying efficiency was due to channeling. The powders elutriated to the top of the bed (collected by filter disc) were sampled at 30 and 60 minutes, and are shown by SEM in Figures 15 and 16. Powder collected at 60 minutes did not appear to be drier than the powder collected at 30 minutes. The newly elutriated, partially dried powders were mixed in with the drier powders and caused the agglomeration of the particles after samples were taken out. With the aid of internals and vibration, all powder elutriated to the top of fluidized bed to form a uniform cake thus eliminated channeling and provided efficient drying. As shown in Figure 17 higher drying gas velocity, 2 m/s, the 20wt% mannitol was dried to porous powder (0.3 wt% residual moisture) at much higher rate than the lower drying gas velocity, 0.39 m/s. The figure shows the effect of flow-rate

on sublimation time in a vibrational fluidized-bed SFD process. With vibration and internals, all of the powder elutriated to the top of the drying bed. A high flow rate resulted in faster drying time and thus higher efficiency.

Example 12 – Mass Transfer Analysis of The Drying Process in a Fluidized Bed and a Fixed Bed Dryer

[000154] The terminal settling velocity of a 20 μm particle is about 0.012 m/s

$$(\text{velocity} = \frac{\rho_p d_p^2 g}{18\mu}), \text{ which corresponds when elutriation occurs for a single frozen}$$

particle. At such low gas flow rate (low Reynolds number) the mass transfer coefficient is very low. Richardson and Szekely (1961) have established an empirical equation below to correlate the mass transfer coefficient, k , with the Reynolds number for a gas-solid fluidized-bed.

$$Sh = 0.37 Re^{1.8} \quad 0.1 < Re < 15,$$

where Sh is Sherwood number ($=kd_p/D$), k is mass transfer coefficient, D is diffusion coefficient, d_p is particle size), Re is Reynolds number ($=\rho_g u d_p / \mu$).

If particle agglomerate size $d_a=200 \mu\text{m}$, nitrogen density 1.29 kg/m^3 , viscosity of nitrogen fluid $1.81 \times 10^{-5} \text{ kg/m/s (Pa}\cdot\text{s)}$, fluid velocity 0.012 m/s (the terminal velocity of single particles at 20 μm . At the fluidizing gas velocity higher than the terminal velocity of single particles, the single particles derived from the collision and attrition of agglomerates in fluidized bed will be carried away or elutriated), ($Re=0.171$) then Sh is 1.54×10^{-2} .

[000155] At drying gas velocity greater than 0.012 m/s, the particles (20 μm or smaller) will elutriate out of the fluidized-bed and need to be collected by a filter or a cyclone. The operations by Leuenberger and BDT are far beyond the terminal velocity of the particles and a filter disc collects the powders as the cold dry gas stream continuously dries the frozen powders. In effect, the wet-frozen powders are elutriated and form a uniform cake at the top exit of the fluidized bed. As the drying gas is flowing through at high speed, the drying process is similar to that of a fixed-bed dryer. The mass transfer in a fixed bed can also be calculated from another empirical equation proposed by Ranz (1952) *Chem. Eng. Prog.* 48, 247:

$$Sh = 2.0 + 1.8Sc^{\frac{1}{3}} Re^{\frac{1}{2}}$$

where Sc is Schmidt number ($=\mu/\rho_g D$). At high Re , for example, if particle size is 20 μm , nitrogen density 1.29 kg/m^3 , viscosity of nitrogen fluid 1.81×10^{-5} kg/m/s , fluid velocity 2.0 m/s (60 liter per minute in our experiment, pressure of source nitrogen 40 psi), then the Sh is calculated to be 4.7. By comparing the mass transfers between gas and solids in a fluidized bed at low Re and a fixed bed at high Re , the ratio of mass transfers in two situations is about 1:300.

[000156] In one set of experiments, the drying nitrogen velocity was at 0.03 m/s to dry 5wt% PEG solution (95% moisture). At this flow rate, there were some powders elutriated (and collected by filter paper). However, no "powder" was observed on the filter paper since these frozen particles were not fully dried yet and thawed out as they deposited on the filter paper. The fluidized frozen sample collected at the bottom of the bed (truly fluidized) after 4 hours still contained 93% moisture. This observation supports our Sherwood number analysis that slow fluidization gives rise to poor mass transfer (drying) rate.

Example 13-Recombinant Staphylococcal Enterotoxin B Vaccine in Dry Powder Form

[000157] Novel aerodynamically light powder (ALP) formulations of a mutated form of Staphylococcal Enterotoxin B, produced recombinantly (rSEB), can be used to vaccinate against toxic shock syndrome, which occurs on exposure to pathogens such as *Staphylococcus sp.* These ALP vaccine formulations show evidence of being more powerful in protecting mice compared to the standard liquid rSEB formulation.

Sample Preparation

[000158] Aerodynamically Light Powder (ALP) formulations of rSEB vaccine were prepared by the method described above in Example 2. Briefly, a solution of the protein in a PBS sucrose mixture was nebulized using an Accuspray™ nozzle, into a liquid nitrogen bath. The frozen particles thus formed were then lyophilized in a Vertis lyophilizer to remove moisture. Samples prepared by this method are referred to as spray-freeze-dried (SFD).

[000159] The preparation of SFD rSEB for the potency assay and other analytical work is described below.

[000160] SFD Procedure:

1. Dissolve 200 mg of sucrose in 2 mL of DI water.
2. Thaw two vials of SEB (approximately 2 mL total volume).
3. Mix SEB with sucrose solution in a 5-mL Accuspray™ syringe.
4. Mixed solution is sprayed into liquid N₂ according one of the methods described herein.
5. Cool all necessary materials (spatula, scintillation vial, lyophilizer container, etc) in dry ice to minimize heat transfer when collecting particles.
6. Collect frozen SFD particles and place in a scintillation vial.
7. Place scintillation vial in lyophilization chamber and attach chamber to a manifold lyophilizer. Dry ice should be placed around the lyophilizer container to ensure that the SFD particles stay frozen throughout primary drying.

[000161] In addition, powder samples were also prepared by standard lyophilization methods. After lyophilization, samples were milled according to the following procedure: Aliquots of powder samples are placed in a 1 ml stainless steel vial with a grinding ball until vials are two-thirds full by volume. The vial is placed in a reciprocating Wig-L-Bug mill model 3110-37A and milled for 30 minutes per aliquot until all samples are milled to the appropriate particle size. After milling, each aliquot is removed by spatula and placed in a vial for further use.

Potency Assay

[000162] A USAMRIID (United States Army Medical Research Institute for Infectious Disease, Fort Detrick, MD) potency assay was used to determine the survival rates for test groups of mice challenged with wild type SEB after parenteral vaccination with either standard rSEB solutions or reconstituted SFD rSEB at 5 and 20 ug/mouse. The challenge doses were 2 and 15 ug/mouse. SFD rSEB was reconstituted and injected into mice intramuscularly. Reconstitution was carried out as follows: a 60 mg quantity of SFD rSEB powder (containing 5 mg rSEB) was weighed out into a vial. 2.5 ml of 10 mmol/L phosphate buffer solution was

added to the vial, resulting in a clear solution. A detailed protocol for the USAMRIID potency assay for the Recombinant Staphylococcal Enterotoxin B (rSEB) Vaccine is outlined below:

[000163] The following materials and equipment were utilized in the potency bioassay: Mice, female, 250 animals, 8-week-old (minimum), BALB/c, Charles Rivers Laboratories, or equivalent; rSEB vaccine, 1 mg/mL, (reference: Mary A. Woody, Teresa Krakauer, Robert G. Ulrich, and Bradley Stiles, 'Differential Immune Responses to Staphylococcal Enterotoxin B Mutations in a Hydrophobic Loop Dominating the Interface with Major Histocompatibility Complex Class II Receptors,' J. Infect. Diseases, 1998; 177:1013-1022). The control sample for this study was the standard, liquid vaccine described in this reference. SEB (toxin) at a concentration of 1 mg/mL, was obtained from United States Army Medical Research Institute for Infectious Disease, Ft. Detrick, MD (USAMRIID). Other materials include Alhydrogel at 2% aluminum hydroxide adjuvant, Superfos Biosector a/s, or equivalent; Lipopolysaccharide (LPS), Difco Laboratories, BE. coli 055:B5, lipid A 13.7%, or equivalent; Milli-Q water; 50 mM Glycine buffer; Glycine, Bio-Rad, Cat. No. 161-0718, or equivalent; Sodium Chloride; Sodium Hydroxide; 10 mM phosphate buffered saline (PBS), pH 7.4, Sigma, Cat. No. 1000-3, or equivalent; 0.5 inch, 27 gauge needle syringes, capable of delivering 0.1mL doses, Becton Dickinson, Cat. No. 309623, or equivalent; Pipette tips RI00, R200, and RI000 Pipet-Plus pipettors, Rainin, or equivalent; 25 mL disposable serological pipettes; Sterile 15-ml conical tubes; Sterile Glass Bottle, capable of holding 100 mL; Laminar flow hood, Sterilgard, or equivalent; Pipet-Plus Pipettors, R100, and R1 000, Rainin, or equivalent. Pipet-Aid pipettor, Drummond, or equivalent; Millipore water purification system, MILLI-Q, or equivalent and pH meter, Corning, model 240, or equivalent. For statistical analysis SAS Version 8.0 statistical program was used

A. Set-up

[000164] Breakdown of mouse groups and immunization & challenge doses, and dates, was as follows in Table 5.

Table 5

Mouse Group	Vaccine dose ($\mu\text{g/mL}$) / day	Challenge dose ($\mu\text{g/mL}$) / day
1	200 / day 0 & 21	200/ day 31
2	200 / day 0 & 21	150/ day 31
3	200 / day 0 & 21	100/ day 31
4	200 / day 0 & 21	75/ day 31
5	200 / day 0 & 21	50/ day 31
6	200 / day 0 & 21	25 / day 31
7	200 / day 0 & 21	12.5/ day 31
8	200/day 0 &21	6.25 / day 31
9	50 / day 0 & 21	75/ day 31
10	50 / day 0 & 21	50 / day 31
11	50/day 0 &21	25 / day 31
12	50/day 0 &21,	12.5/ day 31
13	50/ day 0 & 21	6.25 / day 31
14	50/day 0 &21	5 / day 31
15	50 / day 0 & 21	2.5/ day 31
16	50/day 0 & 21	1.25 / day 31
17	0	25 / day 31
18	0	12.5/ day 31
19	0	6.25 / day 31
20	0	5 / day 31
21	0	2.5/ day 31
22	0	1.25 / day 31
23	0	0.6 / day 31
24	0	0.3/ day 31
25	0	0 / day 31

[000165] Note: Each group / cage (1-25 in above table) contains 10 mice. Day 0 represents the first day of the assay. Mice are acclimated to current surrounding for not less than 3 days after arrival to laboratory and be released by a veterinarian before any injections are scheduled.

B. Serum Collection

[000166]

1. Serum is collected from each mouse 1-3 days prior to the first vaccination ($t=0$) and the challenge date ($t=21$ days).
2. Serum is tested using ELISA to quantitate mouse antibodies/titer for correlation to protection against SEB.

C. Vaccine solution preparation:**[000167]**

1. All solutions are prepared in a laminar flow hood, using sterile technique. Label all tubes with a unique identifier, date, and rSEB. Vaccine solution preparation is performed on day 0 (first day of assay) and on day 21.

2. Prepare the vaccine solutions, shown below, using 1mg/mL rSEB, 2% Alhydrogel® adjuvant, and 50 mM glycine buffer.

Vaccine solution ID	Vaccine solution mL	Vaccine Dose/ mouse	rSEB vaccine (ml)	Adjuvant (ml)	Glycine Buffer (ml)
200	200	20	2.00	0.71	7.29
50	50	5	0.50	0.18	9.32

[000168]

a. In a 15-ml sterile, conical tube, add together 7.29 mL glycine buffer, 0.71mL adjuvant, and 2.00 mL rSEB. Label with a unique identifier.

b. In a 15-mL sterile, conical tube, add together 9.32 mL glycine buffer, 0.18 mL adjuvant, and 0.50 mL rSEB. Label with a unique identifier.

c. Both vaccine solutions will be kept at room temperature (RT) and used within 3 hours.

D. Vaccination procedure**[000169]**

1. Mix vaccine solutions by gently inverting tube 5 times before loading each syringe.

2. Load the sterile syringes with 1.0 mL of 200 µg/mL vaccine solution. Remove any air bubbles.

3. Inject each mouse from group 1 through 8 with 0.1 mL, intramuscularly, in thigh.

4. Load the sterile syringes with 1.0 mL of 50 µg/mL vaccine solution.

5. Remove any air bubbles.
6. Inject each mouse from group 9 through 16 with 0.1 mL, intramuscularly, in thigh.
7. Dispose used syringes in appropriate sharps container and unused vaccine solutions by the end of day.
8. Repeat vaccination procedure on day 21 of assay by preparing fresh vaccines and injecting animals as previously stated.

E. Challenge solution preparation:

[000170]

1. This step will be performed on day 31 of the assay.
2. Prepare solutions in a laminar flow hood, using sterile technique. Label all tubes with a unique identifier, date, and SEB.
3. Prepare the challenge solutions with SEB (1 mg/mL) and PBS in accordance with the serial dilution schedule shown below in Table 6:

Table 6

Tube No.	Challenge solution (μ /mL)	Challenge dose / mouse (μ g)	SEB (mL)	From Tube	PBS (mL)
1	200	40	2.13		8.50
2	150	.30	8.12	1	2.71
3	100	.20	8.33	2	4.16
4	75	15	10.0	3	3.33
5	50	10	8.32	4	4.16
6	25	5	7.50	5	7.50
7	12.5	2.5	7.50	6	7.50
8	6.25	1.25	7.50	7	7.50
9	5	1	7.36	8	1.84
10	2.5	0.5	4.20	9	4.20
11	1.25	0.25	3.40	10	3.40
12	0.6	0.12	1.80	11	1.95
13	0.3	0.06	1.25	12	1.25
14	0	0	0		1.5

[000171]

- a. In a sterile, 15 ml conical tube, add 2.13 mL SEB to 8.50 mL PBS. The unique identifier for this tube is "1".
- b. Using new, sterile, 15 mL conical tube, add 8.12 mL challenge solution (removed from tube 1) and 2.71 mL PBS. The unique identifier for this tube is "2".
- c. Continue following the serial dilution schedule of the challenge SEB until all 14 tubes have been completed.
- d. All challenge solutions will be used within 3 hours and require no storage. Unused challenge solution will be disposed by the end of day.

F. Challenge procedure:**[000172]**

1. The time of challenge for each group of must be noted on Results form for the purpose of the LPS potentiation (4 hours post challenge). The time of challenge will be defined as the time of inoculation of the last animal for each group.
2. Mix challenge solutions by gently inverting tube 5 times before loading each syringe.
3. For mouse groups vaccinated with 200 $\sim\mu$ g/mL (Group 1-8, 20 $\sim\mu$ g/mouse)

- a. Use challenge solutions 200, 150, 100, 75, 50, 25, 12.5 and 0.25 µg/ml
 - b. Load a sterile syringe with 1.0 mL of 200 µg/mL challenge solution.
Remove any air bubbles.
 - c. Inject 0.2 mL, intraperitoneally, into each of the 10 mice per group. Two sterile syringes will be needed for each of the 8 groups.
 - d. Continue step 3b and 3c until all of the eight challenge solutions have been administered
4. For mouse groups vaccinated with 50 ug/mL (Group 9-16, 5 µg/mouse):
- a. Use challenge solutions 75, 50, 25, 12.5, 6.25, 5, 2.5, and 1.25 µg/mL.
 - b. Load a sterile syringe with 1.0 mL of 75 µg/mL challenge solution.
Remove any air bubbles.
 - c. Inject 0.2 mL, intraperitoneally, into each of the 10 mice per group. Two sterile syringes will be needed for each of the 8 groups.
 - d. Continue step 4b and 4c until all of the 8 challenge solutions have been administered.
5. For standard curve mouse groups (17-25):
- a. Use challenge solutions 25, 12.5, 6.25, 5, 2.5, 1.25, 0.6, 0.3 and 0 µg/mL
 - b. Load a sterile syringe with 1.0 mL of 25 µg/mL challenge solution.
Remove any air bubbles.
 - c. Inject 0.2 mL, intraperitoneally, into each of the 10 mice. Two sterile syringes will be needed for each of the 8 groups.
 - d. Continue step 5b and 5c until all of the 9 challenge solutions have been administered.
6. Dispose of the used syringes in appropriate sharps container and the unused challenge solutions by the end of day.

G. LPS potentiation solution preparation:

[000173]

1. The LPS potentiation solution is prepared on the day of use and stored at 4±2°C.
2. The LPS potentiation solution must be prepared in a laminar flow hood, using sterile technique.

3. Prepare the LPS solution as shown below; using 5 mg/mL LPS and PBS

LPS solution	LPS Dose / mouse	LPS (mL)	PBS (mL)
200 µg/mL	40	2.2	52.8

- a. In a sterile glass 100 mL bottle, add 2.2 mL LPS and 52.8 mL PBS. Label with the date, initials or technician. and "LPS".

H. LPS potentiation procedure:

[000174]

1. This step must be started 4 hours (\pm 15 minutes) after the start of the challenge procedure.
2. Mix LPS solutions by gently inverting tube 5 times before loading each syringe.
3. Load a sterile syringe with 1.0 mL LPS solution. Remove any air bubbles.
4. Inject 0.2 mL, intraperitoneally, into each of ten mice per group. Two sterile syringes will be needed for each group of mice.
5. Repeat step H2 - H3 until all 250 mice have received 0.2 mL of LPS solution.
6. Dispose of used syringes in appropriate sharps container and the unused LPS solution by the end of day.
7. Monitor cages once each 24 hours and remove any dead mice. At 72 hours (\pm 1 hour) after LPS injections, note the number of surviving mice in each cage.

Acceptance Criteria:

[000175]

The survival rates for both test groups (challenge doses 2 and 15 µg SEB/mouse) are compared to the standard by Fisher exact test using SAS version 8.0. If there is a difference between any group and its standard, a p-value reflecting this at the 95% confidence level would be less than 0.05. This portion of the assay conforms to standard if the p-value comparing the test group and the standard is above 0.05.

[000176]

The controls (0 µg rSEB/mouse) are analyzed by probit analysis using SAS version 8.0.

The estimated LD50 (challenge dose) with 95% confidence limits and probability of survival are calculated. At the estimated LD50, 50% of the mice are expected to survive. This portion of the assay conforms to standard if all challenge doses used in the assay fall within their predicted probability of survival ($\pm 20\%$).

Light Scattering

[000177]

A Wyatt Dawn EOS light scattering instrument (Wyatt Technology Corp., Santa Barbara CA) was used to obtain molecular weight, radius of gyration and hydrodynamic radius for unprocessed liquid, and reconstituted SFD and lyophilized samples.

FTIR

[000178]

FTIR measurements were made to estimate the storage stability of the powders using the methods developed by John Carpenter and others (J. F. Carpenter, S. D. Allison, and S. J. Prestrelski. Infrared Spectroscopic Studies of Lyophilization-Induced Protein Aggregation. *Abstracts of Papers of the American Chemical Society* 207:94-BIOT, 1994; J. F. Carpenter, M. J. Pikal, B. S. Chang, and T. W. Randolph. Rational design of stable lyophilized protein formulations: Some practical advice. *Pharmaceutical Research* 14 (8):969-975, 1997; J. F. Carpenter, S. J. Prestrelski, and A. C. Dong. Application of infrared spectroscopy to development of stable lyophilized protein formulations. *European Journal of Pharmaceutics and Biopharmaceutics* 45 (3):231-238, 1998; A. C. Dong, S. J. Prestrelski, S. D. Allison, and J. F. Carpenter. Infrared Spectroscopic Studies of Lyophilization-Induced and Temperature-Induced Protein Aggregation. *Journal of Pharmaceutical Sciences* 84 (4):415-424, 1995; S. J. Prestrelski, N. Tedeschi, T. Arakawa, and J. F. Carpenter. The Structure of Proteins in Lyophilized Formulations Using Fourier-Transform Infrared-Spectroscopy. *Abstracts of Papers of the American Chemical Society* 205:145-BIOT, 1993.)

[000179] Powders were mixed with KBr in a standard pellet for transmission infrared spectroscopy. The second derivative of the amide peaks gives an estimate of the amount of physical aggregation in the sample.

[000180] The experiments were conducted as follows:

Materials:

Liquid "as received" rSEB vaccine (non-powder-processed) was obtained from USAMRIID, at 10 mg/ml in phosphate buffered saline (see above potency protocol).

Lyophilized rSEB vaccine was prepared by mixing the "as received" vaccine with pharmaceutical grade sucrose powder at a ratio of one part rSEB to 10 parts sucrose (w/w) to obtain a 10 mg/ml solution of rSEB with sucrose. The solution was lyophilized in a bench dryer for 3 days and milled as described above. ALP rSEB vaccine was produced by preparing a solution of rSEB with sucrose as described above, then spray-freeze-drying the solution using an Accuspray™ system.

FTIR Protocol:

[000181] To determine the stability of the rSEB protein antigen after processing, 2nd derivative FTIR spectra were obtained of the liquid sample using a special short pathlength cell. This spectrum was used to compare the spectra obtained using standard KBr pellets of the solid lyophilized sample and the solid ALP sample. All peaks have been normalized by area, therefore the relative areas of different band reflect the relative proportion of that particular feature in the sample.

ELISA

[000182] Antibody panels can be used to monitor proteins for structural changes. In this study the SFD rSEB was compared to lyophilized rSEB and unprocessed rSEB ("As Received") from USAMRIID.

Antibody Selection

[000183] Seven commercial monoclonal antibodies specific for SEB were prescreened by Western and ELISA for their ability to provide information on structural changes.

[000184] In the Western prescreen, rSEB was loaded in multiple lanes and electrophoresed under reducing conditions. The protein was transferred to a 0.2-micron nitrocellulose membrane. Each lane was subsequently probed with one of the commercial antibody candidates.

The transfers were then exposed to a secondary Goat Anti-Mouse Ig alkaline phosphatase conjugate and developed using NBT/BCIP substrates.

[000185] There were two parts to the ELISA prescreen. In the first ELISA screen, each antibody was titrated in a 96-well Nunc Maxisorb plate coated with unprocessed rSEB to identify a dilution producing an OD signal of at least 1.0 and a S:N of 10:1. This process eliminated 2 antibody preps due to concentration. The five remaining antibodies were adequately concentrated and western positive. In the second ELISA screen, the five remaining antibodies were incubated with unprocessed rSEB exposed to three treatments known to induce structural changes. One stock of unprocessed rSEB was exposed to 95 degrees C for 3 minutes to disrupt hydrophobic bonds. Another stock of rSEB was exposed to 0.1% SDS to disrupt ionic bonds. A third stock of rSEB was exposed to both detergent and heat. At least three different signal profiles were observed. The signal from one of the antibodies (2B) was not affected by any of the three treatments. The signal produced by two of the antibodies (3B, 18B) was altered by the detergent treatment. The signal produced by the two final antibodies (3M, S5) was lowered significantly by the combination of heat and detergent. The result obtained with the 2B antibody was essential to carrying out the antibody-based analyses in a half-sandwich format as the antibody provided a means to confirm treated rSEBs were adhering to plate surfaces with similar efficiencies. Note: combining a reducing reagent with the other treatments did not yield a fourth signal profile. An antibody to thyroid stimulating hormone (TSH) was used as a negative control throughout.

Assay

[000186] Having four antibody reagents capable of detecting structural changes and one antibody that could be used to verify coating efficiencies, ELISA plates were coated with rSEB ("As Received"), lyophilized rSEB and Spray Freeze Dried rSEB. Plates were blocked with 10mM Phosphate Buffered Saline + 0.2% Casein then allowed to react with control and probe antibodies. The assay included at least 20 replicates for each rSEB-Antibody combination, and the assay was repeated on four separate days.

Results

Potency Assay

[000187] The results of the potency assay are shown below in Table 7. Briefly, the mice receiving the SFD rSEB consistently showed higher survival rates versus those receiving the standard, non ALP processed rSEB. In addition, serum titers were consistently higher with the SFD rSEB groups. These results demonstrate the superior potency achieved through SFD processing.

[000188]

Table 7

Plate #	Mouse group				Serum dilution	Group SD
		Challenge Dose (ug SEB / mouse)	Alive	group size	1 :10000 [mean o.d.'s (n=3)]	
1	USAMRIID Standard 20ug/mouse Group 1	15.00	6	10	1.623	0.058
1	USAMRIID Standard 20ug/mouse Group 2	15.00	8	10	1.787	0.060
1	USAMRIID Standard 5ug/mouse Group1	2.00	5	10	0.569	0.052
1	USAMRIID Standard 5ug/mouse Group 2	2.00	4	10	0.492	0.025
2	SFD rSEB, 2 mg/mL, 20ug/mouse Group1	15.00	9	10	1.866	0.047
2	SFD rSEB, 2 mg/mL, 20ug/mouse Group 2	15.00	7	10	1.817	0.062
2	SFD rSEB, 2 mg/mL, 5ug/mouse Group 1	2.00	8	10	0.713	0.018
2	SFD rSEB, 2 mg/mL, 5ug/mouse Group 2	2.00	7	10	0.792	0.038
	Controls (0 ug vaccine / mouse)					
	1	5.00	1	10		
	2	2.50	3	10		
	3	1.25	4	10		
	4	1.00	0	10		
	5	0.50	0	10		
	6	0.25	4	10		
	7	0.13	8	10		
	8	0.06	10	10		
	9	0.00	10	10		

[000189] Blank Mean o.d.'s for plates 1 & 2 = 0.0 (Pass) Neg. Cont. Mean o.d.'s for plates 1 & 2 = 0.0 & 0.0 (Pass; samples must have o.d. less than 0.075)

Analysis of rSEB vaccine potency

[000190] The survival rates for groups challenged with 15ug SEB/mouse were compared by Fisher exact test using SAS version 8.2. There are 4 groups in this test, two of which are standards. If there were a difference between any groups and standard, a p-value reflecting this at the 95% confidence level would be less than 0.05. However, the p-value comparing these 4

groups is $p=.6218$. Therefore there is no statistical difference in survival rates between any of the groups challenged at 15ug SEB/mouse.

[000191] A similar analysis for the 4 groups challenged with 2ug SEB/mouse (including two standards) also shows no statistical difference in survival rates at the 95% confidence level ($p=.3118$). Therefore we may conclude that the test articles are not statistically different from standard with respect to survival.

[000192] The controls were analyzed by probit analysis using SAS version 8.2. The estimated LD50 with 95% confidence limits is given in the Table 8 below together with other percentiles of the mortality distribution curve estimated by the probit model. Units are in "ug SEB/mouse" for challenge dose and percentiles are the probabilities of SURVIVAL. Thus, for instance, a challenge dose of 2.93216 ug SEB/mouse is predicted by this model to have a survival rate of .10 (10%), and a dose of 0.30293 ug SEB/mouse to have a survival rate of 50% (LD50), etc.

[000193]

Table 8

Probability	Dose	95% Fiducial Limits	
0.01	18.65951	2.59430	3.29674E41
0.02	11.51337	1.96048	7.92815E35
0.03	8.47532	1.63130	2.17165E32
0.04	6.73086	1.41451	4.55856E29
0.05	5.58031	1.25511	3.03432E27
0.06	4.75734	1.13009	4.27233E25
0.07	4.13625	1.02773	1.02007E24
0.08	3.64926	0.94131	3.61011 E22
0.09	3.25634	0.86661	1.73383E21
0.10	2.93216	0.80086	1.06298E20
0.15	1.89946	0.55378	1.05897E15
0.20	1.34515	0.37746	1.22705E 11
0.25	1.00047	0.23315	60053877
0.30	0.76691	0.11010	87842
0.35	0.59946	0.02522	451.67128
0.40	0.47450	0.00121	15.64724
0.45	0.37846	0.0000121	3.19717
0.50	0.30293	5.90475E-8	1.48290
0.55	0.24248	2.1051E-10	0.93924
0.60	0.19340	5.9546E-13	0.67969
0.65	0.15309	1.2832E-15	0.52444
0.70	0.11966	1.8977E-18	0.41775
0.75	0.09173	1.6239E-21	0.33721
0.80	0.06822	6.086E-25	0.27199
0.85	0.04831	6.0544E-29	0.21594
0.90	0.03130	5.4716E-34	0.16460
0.91	0.02818	3.3028E-35	0.15449
0.92	0.02515	1.5632E-36	0.14433
0.93	0.02219	5.4564E-38	0.13403
0.94	0.01929	1.2857E-39	0.12351
0.95	0.01645	1.7874E-41	0.11263
0.96	0.01363	1.175E-43	0.10119
0.97	0.01083	2.4361E-46	0.08884
0.98	0.00797	6.587E-50	0.07489
0.99	0.00492	1.5609E-55	0.05743

Light Scattering

[000194] Results of light scattering experiments are shown in Table 9.

Table 9: SEB Light Scattering Results

MW results (g/mol)

Name	Aggregate 1	Aggregate 2	SEB
Unprocessed (as received)	4,807,000	40,260	29,220
Lyophilized	47,740,000	169,200	29,950
SFD	40,520,000	6,456,000	33,020

Rg(z) results (nm)

Name	Aggregate 1	Aggregate 2	SEB
Unprocessed (as received)	28.1	65.8	17.1
Lyophilized	30.3	48.6	14.5
SFD	39.9	47.6	16.0

Rh(w) results (nm)

Name	Aggregate 1	Aggregate 2	SEB
Unprocessed (as received)	38.6	59.5	2.9
Lyophilized	NA	1.9	2.5
SFD	95.9	NA	11.8

Rg/Rh Results

Name	Aggregate 1	Aggregate 2	SEB
Unprocessed (as received)	0.73	1.10	5.9
Lyophilized	NA	25.6	5.8
SFD	0.42	NA	1.35

[000195] The calculated molecular weights agreed with the theoretical molecular weight of 30,000. Molecular weight increased with both lyophilization and SFD processing compared with the unprocessed sample. The radii of gyration (Rg) are nearly the same for all samples while the apparent hydrodynamic radius (Rh) increased for the SFD SEB. For the aggregate fractions labeled 'Aggregate 1' in Table 1, the concentrations of aggregates were in the range of 0.8% to 1.6% (w/w) for each of the three samples. For the aggregate fractions labeled 'Aggregate 2' in this table, the aggregates for the unprocessed sample were at a concentration of about 2% (w/w), and about 0.5% (w/w) the two powder processed samples.

FTIR of rSEB Vaccine

[000196] The liquid, non-powder-processed 2nd derivative spectrum is shown in Figure 22. Note that the predominant “twinned” band is present at ~1640 inverse cm. This corresponds to the alpha helical and beta sheet structure present in the protein. No other peaks are dominant in the liquid spectra.

[000197] The spectrum of the lyophilized sample in Figure 23 shows the main 1640 inverse cm band, also. More importantly, it also has a large band at 1690 inverse cm. This corresponds to intermolecular beta sheet structures. Other bands between 1640 and 1690 correspond to some beta turn structures in the protein. One key observation is that the band delineating protein aggregates is larger than that of the band delineating the inherent protein conformation. This demonstrates that the lyophilization process has altered the higher order structure of the protein.

[000198] The spectrum of the ALP sample is shown in Figure 24. Again, the predominant 1640 band is evident. Also, the aggregation peak at 1690 inverse cm is present, however, it is significantly smaller than the peak at 1640 inverse cm. The ALP has also altered the higher order structure of the protein, however, judging by the relative sizes of the two peaks, less aggregates appear to have formed in this sample than in the sample prepared by lyophilization.

ELISA

[000199] The data from ELISA's were normalized, and the means summarized in a bar graph (Figure 25) in a manner that would allow SFD rSEB to be compared with lyophilized rSEB, SFD rSEB with unprocessed rSEB and lyophilized rSEB with unprocessed rSEB. Specifically, the response variable OD450 was first “normalized.” The normalization was performed by dividing the value of each OD450 measurement by the average value for the *native* wells for the plate and for the antibody. These measurements were then converted to percent by multiplying these by 100. The normalized percent is the response variable used in the analysis. The comparison was performed 5 times, once for each antibody probe.

[000200] The statistical design used for the experiment was a split-split plot, and the analysis was performed using SAS version 8.2 statistical design software (SAS Institute, Cary,

NC). The whole plot treatment was the day measurement was obtained, and the whole plot units were the plates (5 plates for each day, giving a total of 20 plates). The split plot treatment was *rSEB: native, Lyoph, and SFD*. The split plot unit is the plate quadrant (4 X 20 = 80 units). The split-split plot treatment was the type of antibody (*C86203M, Mab 18B Anti-SEB, Mab 2B Anti-SEB, Mab 3B Anti-SEB, RDI-TRK2S3-S5*). The split-split plot units are the wells (24 wells per quadrant, or 96 per plate). Note that the *measurement day* variable was treated as a fixed effect, rather than a random effect in the analysis.

[000201] The least-squares estimates of the difference in mean percentages for each pair of the three treatments for each antibody were plotted on the bar graph. The heights of the bars represent the estimated mean differences. The vertical lines passing through the bars represent confidence intervals for the true mean difference. These give a range of plausible values of the true mean difference. Thus, if the confidence interval contains 0, then it is plausible that true mean difference is 0 (i.e., the two means are the same). If the confidence interval does not contain 0, then the data gives strong statistical evidence that the true mean difference is *not* 0. For example, for the antibody *C86203M*, the estimated difference in the mean percentages for *Lyoph* and *Native* is approximately 4.2% (mean normalized percent value for *Lyoph* minus mean normalized percent value for *Native*).

[000202] The data generated with MAB 3B suggest a structural difference may exist between lyophilized and unprocessed rSEB. Likewise RDI-TRK2S3-S5 suggests a difference may exist between unprocessed and SFD rSEB. The data generated with MAB 18B suggests a difference may exist between lyophilized and unprocessed, as well as, lyophilized and SFD rSEB.

[000203] In conclusion, while the antibodies available for the panel were limited, and the estimated differences are small, the combined data suggests at least a subtle change in the native rSEB may be induced by both drying methodologies (lyophilization or SFD).

[000204] The rSEB produced by the SFD process described here leads to a higher potency vaccine, as demonstrated in the mouse study, compared to the non powder processed rSEB, thus offering the potential for dose sparing and improved protection.

[000205] The initial characterization of rSEB SFD vaccine powder formulations by FTIR, ELISA and light scattering approaches indicates a change in antigen structure compared to the non powder processed antigen. In addition, preliminary animal studies demonstrate that these formulations exhibit higher immune potency relative to the conventional liquid formulation.

[000206] From the foregoing description, one skilled in the art can easily ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make changes and modifications of the invention to adapt it to various usage and conditions.

[000207] Without further elaboration, it is believed that one skilled in the art can, using the preceding description, utilize the present invention to its fullest extent. The preceding preferred specific embodiments are, therefore, to be construed as merely illustrative, and not limitative of the remainder of the disclosure in any way whatsoever.

[000208] The entire disclosure of all applications, patents and publications, cited above and in the figures are hereby incorporated by reference.